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Studies of β-lactamase mediated resistance in *Salmonella enterica*

Fiona Mabel Boyle  
BSc (Hons), Master in Biomedical Science  
A thesis submitted for the degree of Doctor of Philosophy

**Supervisors of Research**  
Professor Martin Cormican  
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Discipline of Bacteriology,  
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College of Medicine, Nursing and Health Sciences,  
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June 2012
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<tr>
<td>6-APA</td>
<td>6-aminopenicillanic acid</td>
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<tr>
<td>7-ACA</td>
<td>7-aminocephalosporanic acid</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>AL</td>
<td>Alkaline lysis</td>
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<tr>
<td>ANSI</td>
<td>American National Standards Institute</td>
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<td>BJM</td>
<td>Bush Jacoby Medeiros</td>
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<td>BSAC</td>
<td>British Society of Antimicrobial Chemotherapy</td>
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<td>CI1</td>
<td>Class 1 Integron</td>
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<tr>
<td>CA-SFM</td>
<td>Comité de l’Antibiogramme de la Société Française de Microbiologie</td>
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<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
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<tr>
<td>CIDR</td>
<td>Computerised infectious disease reporting</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
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<td>CMT</td>
<td>Complex mutant TEM</td>
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<tr>
<td>CRG</td>
<td>Commissie Richtlijnen Gevoeligheidsbepalingen</td>
</tr>
<tr>
<td>CT</td>
<td>Cefotaxime</td>
</tr>
<tr>
<td>CT/CTL</td>
<td>Cefotaxime/cefotaxime plus clavulanic acid</td>
</tr>
<tr>
<td>CTX-M</td>
<td>Cefotaximase</td>
</tr>
<tr>
<td>CVRL</td>
<td>Central Veterinary Laboratory</td>
</tr>
<tr>
<td>DAP</td>
<td>Di-aminopimelic acid</td>
</tr>
<tr>
<td>DIN</td>
<td>Deutsches Institut für Normung</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<td>EMEA</td>
<td>European Medicines Agency</td>
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<td>ESBL</td>
<td>Extended-spectrum β-lactamase</td>
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<tr>
<td>ESC</td>
<td>Extended-spectrum cephalosporin</td>
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<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<td>GlcNAc or NAG</td>
<td>N-acetyl glucosamine</td>
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<td>HK</td>
<td>House keeping</td>
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<td>HPA</td>
<td>Health Protection Agency</td>
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<td>HPSC</td>
<td>Health Protection Surveillance Center</td>
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Abbreviations continued

IEF  Iso electric focusing
IMB  Irish Medicines Board
IPTG  Isopropyl- β-D-thiogalactoside
IRS  Inhibitor resistant SHV
IRT  Inhibitor resistant TEM
IS  Insertion sequence
ISCR  Insertion sequence common region
MBL  Metallo β-lactamase
MDR  Multi drug resistance
MIC  Minimum inhibitory concentration
MLST  Multi locus sequence typing
MLVA  Multi locus VNTR analysis
MurNAc or NAM  N-acetyl muramic acid
NRL  National Reference Laboratory
NSCD  Narrow spectrum class D
NTS  Non Typhoid Salmonella
NWGA  Norwegian Working Group on Antibiotics
OXA  Oxacillinase
PBP  Penicillin binding protein
PBRT  PCR based replicon typing
PCR  Polymerase chain reaction
PFGE  Pulsed field gel electrophoresis
PM  Cefepime
PM/PML  Cefepime/ cefepime plus clavulanic acid
S1-PFGE  S1 nuclease pulsed field gel electrophoresis
SDM  Site directed mutagenesis
SGI  Salmonella genomic island
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<td>SHV</td>
<td>Sulfhydryl variable</td>
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<tr>
<td>SRGA</td>
<td>Swedish Reference Group on Antibiotics</td>
</tr>
<tr>
<td>SSA</td>
<td>Sub Saharan Africa</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
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<tr>
<td>STTR</td>
<td><em>Salmonella</em> Typhimurium tandem repeat</td>
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<tr>
<td>TEM</td>
<td>Temoniera</td>
</tr>
<tr>
<td>TZ</td>
<td>Ceftazidime</td>
</tr>
<tr>
<td>TZ/ TZL</td>
<td>Ceftazidime plus clavulanic acid</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Un-weighted pair group method with arithmetic averages</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<tr>
<td>V-AST</td>
<td>Veterinary antimicrobial susceptibility testing</td>
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<td>VNTR</td>
<td>Variable number tandem repeat</td>
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This work is submitted to fulfill the requirements of the degree of Doctor of Philosophy at the National University of Ireland, Galway. No part of this thesis has been previously submitted at this or any other university. Apart from due acknowledgements, it is entirely my own work.

Signed: ____________________  Date: ________________

Fiona Boyle
Dedications and acknowledgments

To my friends and family

Má tú ag lorg cara gan locht béidh tú gan cara

go deo
‘All truths are easy to understand once they are discovered; the point is to discover them’

Galileo Galilei

(1564-1642)
Well in true ‘Fi’ fashion this is not going to be brief…but here it goes….

I made it! This has been the single most challenging experience of my life. And my close friends and family know how much I relish a challenge but oh my goodness has this been a ‘toughie’!!! It has been a journey of self-discovery and the entire PhD process has taught me a lot about myself. I think I am now an eternal optimist, a trait developed through months and indeed years of wishing and finger crossing that my experiments would work out and that it would all tie together in the end. I don’t think I will ever forget the early mornings walking into the lab, opening the incubator door and praying that my Salmonella would have conjugated overnight, that my PCR’s would have worked, that my mutagenesis experiments would have worked. I believe that I became an optimist as I would have given up long ago if I hadn’t!

My PhD journey has been many things- hard, fulfilling, enlightening, educational, fun, painful, and most of all “character building”. I would not have gotten through it or enjoyed it as much if it hadn’t been for so many people. I have made lifelong friends from the Department of Bacteriology and the NSRL. Sandra Galvin, you have been with me on this journey pretty much from the start. You were always a shoulder to cry on and allowed me to give out and rant when things were not going my way you always had a smile on your face and you made my time in the CSI a treasured memory. Our coffee breaks, chats and let’s not forget our so many fun nights out…..“I want chips”!!! To ‘Jo Jo’ King, your positivity helped me so much and your encouraging words have made it possible for me to get this far. At times when I felt like giving up you were always there encouraging me and telling me to stick it out and ‘put the head down’! I haven’t jumped off the balcony yet! Oh to the nights sitting in front of the fire with a glass of wine and a cup of hot water and lemon!! To Juliette, ‘girl’-you have been a rock, you kept me sane, helped me so much, made me laugh when I needed to, hugged me when I needed it, I can never repay you or thank you enough. Thank you so so much. To Carol and Genevieve, What would I have done without our chats and laughs and debating the ways of the world? Again you were both such a positive influence to have around me over the past
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Acknowledgements and dedications

To my family, thank you for being there. You have all put up with seeing very little of me over the past four years…that is all about to change! To Susan, you have been such a support; you have helped me through so much this past few years. To my beautiful god daughter Laya- your laugh and smile always helped me to see the bright side of things. To Hiro….without him I definitely would have cracked up on those evenings writing! You are a star….a truly amazing animal! To Mammy and Daddy…I eventually got there!!! I love you both, thank you for always being there.

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Most of all to Aunty Tessie, we all miss you.

This is for all of you…
This thesis is divided into three sections. Each section focuses on occurrences of β-lactamase/ESBL mediated resistance in Salmonella and the role that plasmids play in the dissemination of same.

Section 1 of this thesis investigated the genetic basis of a novel ESBL phenotype (‘cefepimase’). This phenotype was observed in 19 isolates of S. Typhimurium from Kenya, Ireland and Malawi. All isolates harboured a c. 39kb plasmid termed pFEP39. Plasmid pFEP39-1dr conferred resistance to cefepime with significant inhibition by CA. Sequencing results identified $\text{bla}_{\text{OXA-1}}$ in conjunction with an unusual promoter combination directly upstream of $\text{bla}_{\text{OXA-1}}$. The $\text{P}_2$ promoter was unusual in that there was a ‘GGG’ triplet upstream of the -10 signal. The ‘cefepimase’ phenotype is transferred with a plasmid construct in which $\text{bla}_{\text{OXA-1}}$ was the only β-lactamase gene present.

Section 2 of this thesis investigated ESBL production in seven S. Kentucky isolates from poultry samples in Ireland. S. Kentucky is a common serovar from poultry in Ireland. Four isolates harboured $\text{bla}_{\text{SHV-12}}$ and 3 harboured $\text{bla}_{\text{CMY-2}}$. All 7 isolates harboured 2 plasmids. All isolates were similar but distinguishable by PFGE. This thesis reported the emergence of plasmid-mediated broad-spectrum cephalosporin resistance in S. Kentucky in poultry.

Section 3 of this thesis investigated the comparison of S1 nuclease PFGE and alkaline lysis for analysis of plasmid DNA. The aim was to compare the reproducibility, accuracy and convenience of both the AL and S1-PFGE methods. S1-PFGE was concluded to be more accurate and reproducible than AL. Plasmids were more clearly defined and better separated using S1-PFGE. The S1-PFGE procedure is more convenient to perform than AL and the availability of agarose plugs for other PFGE analysis is an advantage.

The final section of this thesis- the discussion, reflects on the individual chapters.
Chapter 1

Introduction
1.1 The Salmonellae

The genus *Salmonella* contains Gram-negative, non-sporulating bacilli, facultatively anaerobic, oxidase negative, mixed acid fermenting and are motile due to the presence of flagella. The genus comprises a group of clinically important microorganisms that cause a spectrum of disease in a variety of hosts. *Salmonellae* are not part of the normal human micro flora and are usually pathogenic for humans and warm-blooded animals.

1.1.1 Disease caused by Salmonella

*Salmonella enterica* subspecies *enterica* varies in its virulence towards animal species with some serotypes exhibiting a narrow and some a broad host range. Host-specific serovars of *Salmonella* tend to cause more severe systemic illness. Broad host-range *Salmonella* are associated with non-typhoid salmonellosis (NTS) in humans and the host-specific serovars with enteric/typhoid fever. Illness in animals and humans can range from self-limiting bacterial enteric illness (non-typhoidal salmonellosis) to invasive disease (e.g. Typhoid fever caused by *S. enterica* subspecies *enterica* serovar Typhi and paratyphoid fever caused by *S. enterica* serovar Paratyphi A B and C). NTS can lead to long-term health problems such as irritable bowel syndrome and reactive arthritis [1].

In order to cause infection, *Salmonella* must be ingested in sufficient quantities. The infective dose can range from 1-100 colony forming units (CFU’s) [1] with an incubation period of generally 12-72 hours for NTS. Non-typhoid salmonellosis (NTS) in most humans is an uncomplicated gastroenteritis with diarrhoea (in 87% of cases), abdominal pain (in 84% cases), fever (in 75% of cases), nausea (in 65% of cases), muscle pain (in 65% of cases), vomiting and headaches (in 25% of cases) occurring [1].

In approximately 5% of cases of non-typhoid salmonellosis, invasive salmonellosis with bacteraemia can occur [2]. The elderly, infants and immune-compromised individuals are more at risk of developing an invasive *Salmonella* infection. Bacteraemia and septicaemia caused by *Salmonella* presents with fever which may or may not be accompanied by diarrhoea. Complications associated
with invasive Salmonella infection include meningitis, osteomyelitis, and infections in the lungs, kidneys, spleen, endocardium and large blood vessels. In addition, septic arthritis caused by NTS has been reported, however this is quite rare (<1% of NTS cases) [3].

Salmonella cells can continue to be shed in the faeces of children (<5 years) for up to 20 weeks, in adults shedding continues for at least 4-6 weeks and in some cases shedding may occur for prolonged periods (> 1 year) [4]. Chronic carriage of NTS is less common than chronic carriage of Salmonella Typhi [2].

Patients with acquired immune deficiency syndrome (AIDS) are at a greater risk of developing NTS bacteraemia [5]. In blood specimens from AIDS patients in sub-Saharan Africa, (SSA) NTS are the most frequently isolated pathogen. In addition, in 2003 a SENTRY study\(^1\) reported that Salmonella spp. ranked 13\(^{th}\) among the genera most frequently associated with blood stream infections in the cohort of Europe, North America, Asia-Pacific and Latin America [6].

1.1.2 Epidemiology of non-typhoid Salmonellosis

It is estimated that there are approximately 1.3 billion cases of human salmonellosis annually worldwide, with approximately three million deaths [1]. The most common route of transmission in humans is through the consumption of contaminated food. The food products involved in outbreaks can be quite diverse ranging from milk, eggs, poultry, meat, chocolate, potato crisps, spices, bean sprouts and fruit [7].

In 2007 it was estimated that the annual cost of food borne illness (Salmonella data specifically is not available) to the Irish economy (ROI) was €101 million [8]. However, this is not an entirely accurate reflection of the costs to the Irish economy as many cases of food borne illness go unreported. There are no studies presenting a calculation/ multiplier for the correction of under reporting of salmonellosis cases in Ireland. In Australia, for every case of laboratory-

\(^1\) The SENTRY Antimicrobial Surveillance Program (SENTRY) was initiated in 1997 and was designed to monitor the spectrum of microbial pathogens and antimicrobial resistance trends for both nosocomial and community-acquired infections on a global scale, by using validated identification and susceptibility testing methods in designated central laboratories.
confirmed salmonellosis an estimated 7 cases are unreported in the community [9]. Therefore, a multiplier of 7 was applied to estimate the actual number of food borne illnesses attributable to salmonellosis in Australia. In the United Kingdom the most recent multiplier was calculated to be 3.2 [10]. However, in the USA a multiplier of 39 was estimated in 1988 and again in 2004 [11, 12].

In Ireland Salmonella infection is a notifiable disease [13]. Data relating to notifiable diseases are recorded in the computerised infectious disease reporting (CIDR) database. Since 2004 the health protection surveillance centre (HPSC) has monitored, collated and interpreted infectious disease data received from clinicians and laboratory directors within Ireland. In Ireland there are approximately 350-450 cases of salmonellosis reported each year which is a marked decrease compared to the 1990’s, with 1257 cases being reported in 1998 [14]. The reduction in cases of salmonellosis from the late 1990’s to the 2000’s coincides with the trend observed across other European countries. This may be attributed to the introduction of national Salmonella monitoring and control programmes in the late 1990’s [15]. The incidence rate of salmonellosis in Ireland has remained relatively constant since 2000, with approximately 10 cases per 100,000 each year thereafter [14]. Indigenous cases of salmonellosis in Ireland are generally associated with S. Typhimurium infection (38.8% of cases) rather than S. Enteritidis infection (18.6% of cases). S. Enteritidis accounts for the majority of travel-associated infection (43.3%) with S. Typhimurium accounting for 19.7% of travel-associated cases of salmonellosis. However, the country of origin for S. Typhimurium infections was unknown in 33% of recorded cases [14].

In relation to Salmonella in animals intended for human consumption, pork products are considered the most frequent source of transmission of S. Typhimurium in the food chain with 49.4% of Salmonella-positive pork carcasses being contaminated with S. Typhimurium [16]. Salmonella enterica serovars Gallinarum and Pullorum are host-specific serovars that account for the majority of systemic disease in poultry. Colonization of poultry by serovars of
Salmonella can occur by vertical transmission, trans-ovarial transmission \(^2\) through contaminated feed or through the environment. A recent European Food Safety Authority (EFSA) report stated that *S. Typhimurium* is not a common serovar isolated from broilers in the EU \([19]\). *S. Gallinarum* and *S. Pullorum* serovars had been virtually eradicated in poultry production in Europe and the USA by the 1970’s due to the introduction of a test and slaughter system \([20]\). Therefore, nowadays serovars Gallinarum and Pullorum are rarely isolated from poultry. There has been legislation implemented for the reduction of Salmonella serovars in poultry populations in EU member states \([21], [22]\). The purpose of this legislation is to implement control programmes in order to reduce the levels of Salmonella in general in poultry populations and to reduce human cases of salmonellosis.

*S. Enteritidis* is identified in 66.4% of Salmonella positive poultry / egg samples \([23]\). The contamination rates of poultry with Salmonella are 25.4% in Poland, 14.4% in Spain, 10.4% in Portugal, 3.6% in the United Kingdom, 11.2% in Ireland, 14.5% in Germany, 7.4% in France and 2.3% in Switzerland \([19]\). The scale of poultry farming in a particular country is worth considering when analysing these levels. Variation in the level of Salmonella in poultry between countries may be due to factors such as intensity of sampling schedules in a particular country. For example, the EFSA recently reported that Ireland, accounting for only 1.2% of the total number of broilers slaughtered in the EU in 2008, has a prevalence of Salmonella contaminated poultry of 11.2% \([19]\). This is higher than the UK which slaughtered 15.1% of broilers in the EU in 2008 with a prevalence of Salmonella contaminated poultry of 3.6% \([19]\).

A controversial issue in poultry farming is the use of antimicrobials to prevent mass infection in flocks and the use of antimicrobials to inject unhatched eggs to ensure survival of chicks \([24]\). These antimicrobial prescribing practices were highlighted by the Public Health Agency in Canada in response to the growing

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\(^2\) Vertical transmission results from asymptomatic shedding of Salmonella from the intestines of poultry leading to contamination of eggs. Trans-ovarial transmission results in transmission of Salmonella to eggs by contaminated ovaries.
level of cephalosporin-resistant Salmonella isolated from poultry in the country [24].

*Salmonella* Kentucky is the most frequently isolated serovar from poultry in two EU countries- Ireland and the UK [19]. The Central Veterinary Laboratory (CVRL) is the National Reference Laboratory (NRL) for Salmonella in food, feed and animals in Ireland. The CVRL report for 2008 highlighted that *S*. Kentucky was the most frequently isolated Salmonella serovar from poultry carcasses in Ireland, accounting for 72% of poultry isolates [25]. *S*. Kentucky is the third most common serovar isolated from poultry carcasses in the entire EU, after *S*. Infantis and *S*. Enteritidis [19].

*S*. Kentucky was first identified in 1937 from the intestinal tract of a chick in the state of Kentucky, USA [26]. In 2010, the FDA reported that *S*. Kentucky accounted for 45% and 25% of Salmonella isolated from chicken carcasses during commercial processing and from retail chicken breasts respectively [27, 28]. *S*. Kentucky is not a common cause of human salmonellosis cases; the serovar accounted for, on average 62 cases of human salmonellosis per year between 1997-2006 and 123 cases of human salmonellosis in 2006 [28]. In light of this *S*. Kentucky was thought to be a relatively insignificant human pathogen [27-29]. Other common serovars identified from animals were *S*. Seftenberg (3.6%), *S*. Virchow (2.7%) and *S*. Indiana (2.7%) [30].

In Sub-Saharan Africa (SSA) transmission of non-typhoid Salmonella (NTS), particularly certain strains of *S*.Typhimurium may be maintained between humans and not only a zoonotic (foodborne) disease [31]. Up until recently data on incidence rates and epidemiology of NTS in Africa were sparse. The incidence of NTS in humans in different geographical locations within SSA was reported to be 175-388 cases per 100,000 children with an incidence rate of 2000-8500 cases per 100,000 among an adult HIV cohort [32, 33]. However, a recent report by Mackenzie *et al.* into the incidence of NTS in children in Gambia (Fajara and Basse) has shown a decline in numbers [34]. The incidence of NTS in children had fallen from 60-105 cases/100,000 in 1980’s to 10 to
The serovars most commonly associated with salmonellosis are the same as those observed in Ireland and the EU—generally that is serovars Typhimurium and Enteritidis. In Malawi, 75% of NTS cases were attributed to S. Typhimurium with S. Enteritidis accounting for 21% [35]. Other less common serovars (accounting for 2-3% of NTS cases) identified in Malawi were S. Bovismorbificans, S. Choleraesuis, S. Seftenberg, S. Augustenberg and S. Norwich [35].

1.1.3 The application of typing to Salmonella spp.

Discrimination between bacterial isolates is important for outbreak investigation, surveillance, epidemiology, understanding transmission and pathogenesis of disease. The typing methods utilised as part of this thesis are plasmid profiling, pulsed field gel electrophoresis (PFGE), multi locus variable number tandem repeat (VNTR) analysis (MLVA) and multi locus sequence typing (MLST).

Plasmids are extra-chromosomal, double-stranded autonomously self-replicating mobile elements of DNA. Plasmid profiling is valuable in order to categorise plasmids and to analyse their distribution and evolutionary history. The first plasmid typing/ categorisation method was employed by Datta and Hedges in 1971 and was termed ‘plasmid incompatibility’ grouping³. In 1988 Couturier and colleagues developed a plasmid typing scheme based on Southern blot hybridization using cloned replication regions (replicons) of sequenced reference plasmids as probes. A plasmid replicon is defined as the ‘smallest piece of DNA that is able to replicate autonomously and maintain plasmid copy number’. The replicon is a constant genetic unit consisting of an origin of replication and its associated control elements. In plasmids the origin of replication (ori) is a defined segment of DNA several hundred base pairs in length. In 2005 Carattoli and colleagues developed a PCR Based Replicon Typing (PBRT) scheme for the identification of the most common incompatibility groups found in members of

³ Plasmid incompatibility is defined as the inability of two related plasmids to be propagated stably in the same cell line, i.e only compatible plasmids belonging to different incompatibility groups can be recovered from transconjugant cells.
the *Enterobacteriaceae*. However, the most accurate method to determine the plasmid incompatibility group and to characterise the plasmid is ultimately sequencing of the entire plasmid [36].

Pulsed field gel electrophoresis (PFGE) was first described by Schwarz and Cantor in 1984 [37]. PFGE is the most widely used method for molecular typing and epidemiological surveillance of Salmonella. PFGE allows resolution of much larger DNA fragments on agarose gels when compared to standard gel electrophoresis. DNA fragments of ≥40kb in length cannot be sufficiently separated by applying a constant unidirectional electric field as in standard gel electrophoresis. Therefore, regardless of their size, DNA fragments ≥40kb will all migrate at the same speed. This problem is solved by PFGE. Alternating electric fields at different angles are applied to the gel during PFGE. The theory behind alternating electric fields is that larger DNA fragments will take longer to respond to the changes in electric field direction than shorter DNA fragments. With each alteration in the direction of the electric field relative to the gel, shorter DNA fragments will begin moving in the new direction more quickly than the longer DNA fragments. This results in the longer DNA fragment lagging behind, therefore allowing longer DNA fragments (that by conventional electrophoresis would not separate) to be separated efficiently. PFGE of bacterial DNA (e.g. *S. Typhimurium*) begins with immobilisation of a defined concentration of genomic DNA in agarose (called a ‘plug’). The agarose-immobilised cells are then lysed using sodium dodecyl sulphate (SDS) and are subject to a series of washing steps to remove cellular debris. Native DNase, RNase and cellular proteins are degraded by treating the test DNA with proteinase K (a broad spectrum serine protease), hence preventing DNA
degradation during the PFGE procedure. The DNA immobilised in the plug is cleaved using a restriction endonuclease/restriction enzyme, for S. Typhimurium this can be either XbaI or BlnI. A specific banding pattern is generated by this typing method which is termed a Pulsed Field Profile (PFP) and thus enables PFP’s of isolates to be compared for outbreak investigation and epidemiological surveillance. Application of computer software programmes such as BioNumerics (Applied Maths, Kortrijk, Belgium) permits precise size determination of individual bands, thereafter generation of percentage relatedness of isolates based on an algorithm called UPGMA (un weighted pair group method with arithmetic averages) is performed. UPGMA is an algorithm which permits hierarchical clustering with the creation of phylogenetic trees (dendograms). The dendograms generated using this algorithm make outbreak surveillance and identification much more accurate and comparable between laboratories.

The protocols for PFGE for many of the enteric pathogens have been standardised and validated by the Centre for Disease Control (CDC), Atlanta, Georgia [38, 39]. The CDC PulseNet system enables and facilitates this process [40]. The presence of plasmid DNA has implications for interpretation of PFGE banding patterns for test isolates. Larger plasmids can be mistaken for a fragment of cleaved chromosomal DNA. This can be overcome by carrying out plasmid profile analysis alongside PFGE.

Multi Locus VNTR (Variable Number Tandem Repeat) Analysis (MLVA) is a powerful typing tool for sub-typing Salmonella serovars such as S. Typhimurium [41] [42] [43]. MLVA typing schemes have been developed for other serovars of S. enterica including S. Typhi [44] and S. Enteritidis [45]. Linstedt and colleagues, in 2003, recognised the potential for an alternative to PFGE for subtyping S. Typhimurium DT104.
A VNTR is a locus in a genome where short nucleotide sequences are repeated in tandem. A tandem repeat is basically a region of DNA where variations of two or more nucleotides are repeated directly after each other, with variations in length between genomes e.g. AGCTCT- AGCTCT- AGCTCT- AGCTCT- a four tandem repeat of ‘AGCTCT’. VNTR are present in many bacterial species, contain a high level of genetic polymorphisms (i.e. presence of two or more relatively common alleles of a particular VNTR, the more alleles, the greater the polymorphism) and therefore a high discriminatory capability in application to bacterial typing. VNTR evolve at a much faster rate compared to housekeeping gene nucleotide sequences (as targeted in MLST) and this is why they have been identified as an especially useful tool for strain discrimination. When multiple different VNTR’s are targeted for analysis the procedure is called Multi Locus VNTR Analysis or MLVA.

The VNTR are flanked by conserved non repetitive sequences, this allows the specific VNTR locus to be amplified by PCR. The forward primer used for PCR is fluorescently labelled, enabling detection and subsequent size determination of the VNTR by automated high resolution capillary electrophoresis. The use of multiple fluorescently labelled primers allows multiplexing of dispersed target VNTR’s from across a bacteria’s genome, even those that may end up being of similar size. The number of repeats per particular locus in the VNTR typing scheme is calculated by subtracting the known length of the flanking sequence (on each side of the VNTR, termed ‘offset 1’ and ‘offset 2’) from the amplicon length and dividing the result by the known length of each individual VNTR repeat sequence.

In the MLVA typing scheme for S. Typhimurium the VNTR are designated ‘STTR’, this is an acronym for Salmonella Typhimurium Tandem Repeat. There are 5 STTR analysed in MLVA of S. Typhimurium: STTR3 (of which there are
two variations- 27 bp and 33bp repeats), STTR5 (6 bp repeats), STTR6 (6 bp repeats), STTR9 (9bp repeats) and STTR10pl (‘pl’ means these loci are located on a serovar specific plasmid pSLT and consist of 6 bp repeats) The order in which they are reported is always in the form of the allelic string- STTR9-STTR5-STTR6-STTR10pl-STTR3 [46] [41]. The loci are amplified by multiplex PCR using primers incorporating fluorescent dyes. Once the amplification is complete the size of the fluorescently labelled amplicons can be analysed more efficiently and accurately using a DNA sequencing machine. The MLVA allelic profile/ string once constructed can be entered into Bionumerics system (Applied Maths, Kortrijk, Belgium) for analysis.

There is a standard approach to MLVA in Europe for S. Typhimurium, with Larsson and colleagues recently suggesting a nomenclature in order to allow simple and rational assignment of MLVA profiles [43]. The Institut Pasteur in Paris is in the process of trying to harmonise existing MLVA databases [47] in use at present by the introduction of a system [48] allowing storage of the raw data as well as the analysis tools provided by other websites such as: http://mlva.u-psud.fr/, http://www.mlva.eu and http://www.miru-vntrplus.org .

Multi Locus Sequence Typing (MLST) is another typing tool available. MLEE (Multi Locus Enzyme Electrophoresis) is an epidemiological typing tool that was the phenotypic predecessor of MLST. With MLEE analysis, enzyme polymorphisms are detected between test strains by analysing the different electrophoretic abilities of a variety of cellular enzymes on a gel. A panel of enzymes are chosen to use in a particular MLEE typing protocol. Each enzyme is analysed according to its electrophoretic mobility on a starch gel. The rates of migration are determined by using enzyme standards and staining solutions that include enzyme specific substrate, co-enzymes, cofactors and dyes. Differences in electrophoretic mobility of the same enzyme amongst a set of test study bacterial isolates results when there is a difference in charge of the enzyme caused by substitutions in its amino acid coding region. This infers changes in the underlying DNA encoding the polypeptide. Each target enzyme in each study bacterial isolate is allocated a number according to its electrophoretic mobility.
Therefore, each study isolate will have a series of numbers which act as a fingerprint or MLEE profile. The major draw back with MLEE was that it determined phenotypes not genotypes.

Isolate characterisation by multi locus sequence typing (MLST) is based on the determination of the DNA sequence in a predefined set of housekeeping, ribosomal or virulence genes for isolate characterisation. Isolate characterisation by MLST is based on the determination of the DNA sequence in a predefined set of housekeeping, ribosomal or virulence genes. It was developed in 1998 by Maiden and colleagues using the human pathogen \textit{Neisseria meningitidis} as a model organism \cite{49}. MLST is capable of identifying all nucleotide sequence changes, including nucleotide sequence changes that do not necessarily result in amino acid changes. The frequency of these synonymous changes\footnote{A ‘silent’ substitution, a nucleotide change that does not result in a change in the amino acid sequence} can also be used to estimate the evolutionary timescale of bacterial groupings by comparison with a molecular clock rate \cite{50-52}. The molecular clock hypothesis is a method of analysing rates of molecular change in order to be able to estimate divergence within a population (i.e. species, taxa). The molecular clock is also referred to as an ‘evolutionary clock’.

An MLST scheme for sub-typing \textit{S. enterica} was set up by Kidgell and colleagues in 2001. This typing scheme uses seven house keeping genes as targets for analysis (\textbf{Table 1.1}) \cite{50}. The MLST process is outlined in \textbf{Figure 1.1}.\footnote{A ‘silent’ substitution, a nucleotide change that does not result in a change in the amino acid sequence}
Table 1.1 MLST housekeeping genes utilised for MLST of *Salmonella enterica*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size of PCR product</th>
<th>MLST internal fragment size</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>thrA</td>
<td>852bp</td>
<td>501bp</td>
<td>Aspartokinase &amp; homoserine dehydrogenase</td>
</tr>
<tr>
<td>purE</td>
<td>510bp</td>
<td>399bp</td>
<td>Phosphoribosylaminomisazole carboxylase</td>
</tr>
<tr>
<td>sucA</td>
<td>643bp</td>
<td>501bp</td>
<td>Alpha ketoglutarate dehydrogenase</td>
</tr>
<tr>
<td>hisD</td>
<td>894bp</td>
<td>501bp</td>
<td>Histidinol dehydrogenase</td>
</tr>
<tr>
<td>aroC</td>
<td>826bp</td>
<td>501bp</td>
<td>Chorismate synthase</td>
</tr>
<tr>
<td>hemD</td>
<td>666bp</td>
<td>432bp</td>
<td>Uroporphyrinogen III cosynthase</td>
</tr>
<tr>
<td>dnaN</td>
<td>833bp</td>
<td>501bp</td>
<td>DNA Polymerase III beta sub unit</td>
</tr>
</tbody>
</table>
Figure 1.1 MLST workflow utilising Bionumerics software, (Applied Maths, Kortrijk, Belgium)

1. **PCR amplification of seven housekeeping (HK) genes**
2. **Sequence internal fragment of HK gene**
3. **Trace file/ chromatogram generation by sequencing machine**
4. **Import trace file to Bionumerics**
5. **Automatic identification of strain number and gene name from trace file name**
6. **Automatic forward and reverse strand trimming and assembly**
7. **Detailed quality report generated by Bionumerics using colour codes for query nucleotides**
8. **Quality checked sequences assembled and passed by Bionumerics system**
9. **Query sequences manually edited**
10. **Enter consensus sequences into MLST database to generate the allelic number and thereafter the allelic string for each study isolate**
11. **Sequence Type (ST) for the study isolate determined, enter into MLST database**
According to the MLST database (central MLST database) hosted at http://pubmlst.org/databases.shtml, as of 09/03/2012 there are 1554 sequence types (STs) and 4892 isolates of *Salmonella enterica* available for comparative analysis. This data is stored and maintained by the Environmental Research Institute (ERI) at University College Cork on the *S. enterica* MLST database [53]. MLST is very valuable in evolutionary analysis of bacteria [54], however MLST may be less discriminatory than PFGE for subtyping [55].

1.2 Antimicrobial agents

1.2.1 An introduction to antimicrobial agents and mechanisms of action

Antimicrobial therapeutic agents play a major role in clinical, veterinary and agricultural sectors. They can be classified as (a) original natural products, (b) products derived or chemically synthesised from natural products or (c) chemically synthesised products. Natural antimicrobials or antibiotics are produced by a wide variety of bacteria and fungi enabling them to kill or inhibit growth of other microorganisms. The term ‘antimicrobial’ will be used throughout this thesis to include all of these agents.

The ‘antibiotic era’ began in 1928 with Alexander Fleming’s discovery of an antimicrobial agent which inhibited the growth of *Staphylococcus aureus*—this antimicrobial, produced by the fungus *Penicillium notatum* and was called penicillin G. In 1945 penicillin was introduced into clinical practice, and subsequently other, naturally-occurring antimicrobials such as erythromycin and streptomycin were identified.

The four major bacterial cell targets of commonly used antimicrobial classes are:

- The bacterial cell wall: e.g. β-lactams and glycopeptide’s
- Bacterial protein synthesis: e.g. tetracyclines, macrolides, glycyclcyclines, aminoglycosides and oxazolidinones.
- Bacterial DNA repair and synthesis mechanisms: e.g. quinolones and fluoroquinolones.
• Bacterial metabolic pathways: e.g. sulphonamides inhibit the bacterial folate co-enzyme pathway.

1.2.2 The cell wall

The differences between the cell wall architecture of Gram-positive and Gram-negative bacteria hinder the penetration of certain antimicrobials into the bacterial cell. The Gram-positive bacterial wall is more easily penetrated than its Gram-negative counterparts as the cell is surrounded by a single layer of peptidoglycan that is essential in maintaining bacterial cell structure and integrity. The Gram-negative cell has this wall and an outer membrane layer termed the lipopolysaccharide layer (LPS). The peptidoglycan layer in both Gram-positive and Gram-negative bacteria fundamentally consists of orthogonal (intersecting and lying at right angles) glycan and peptide strands.

The peptidoglycan layer is comprised of alternating chains (glycan chains) of the monosaccharides- N-acetyl glucosamine (GlcNAc or NAG) and N-acetyl muramic acid (MurNAc or NAM) which are both derivatives of glucose. GlcNAc and MurNAc units are bound together by β 1-4 glycosidic bonds. The β 1-4 glycosidic bonds are formed through the action of the enzyme transglycosidase. In Gram-negative bacteria MurNAc contains a pentapeptide side chain consisting of the amino acids- L-alanine, d-alanine, D-glutamic acid and Diaminopimelic acid (DAP). The terminal portion of the precursor pentapeptide side chain is made up of a repeat D-alanine-D-alanine segment. In Gram-positive bacteria the difference is L-Lysine instead of DAP. The linear peptide side chains are cross linked by a interpeptide bond in Gram-negative bacteria and an interpeptide bridge in Gram-positive bacteria. This occurs by a bond forming between the carboxyl group (-CO-) of D-alanine and the amino group (-NH-) of DAP (Gram-negative) or L-Lysine (Gram-positive). In Gram-positive bacteria the interpeptide bridge is formed by a peptide consisting of five glycine molecules. This is a pentaglycine bridge. The interpeptide bridge/bond is formed through the action of the enzyme transpeptidase. The terminal D-alanine-D-alanine is displaced from the carboxy terminus of the peptide chain prior to cross linking of the penultimate D-alanine with either DAP or L-lysine. It is this
rigid cross linking of the peptidoglycan structure that provides the front line structural barrier in preventing lysis of the bacterial cell.

Autolysins are the enzymes that are responsible for degrading the β 1-4 glycosidic bonds between GlcNAc and MurNAc units. In this way the MurNAc and GlcNAc units can be added to a growing peptidoglycan chain. Therefore, growth and division of the bacterial cell can occur.

1.2.3 β-lactam antimicrobial agents
β-lactam antimicrobials are versatile and today represent more than 60% of antimicrobial usage [56]. This is due to their safety and efficacy and their potential to be chemically modified to optimise antimicrobial activity. The β-lactam antimicrobials consist of the penicillins, cephalosporins, cephemycins, monobactams and carbapenems. Members of the β-lactam class are characterised by a four-carbon ring structure.

β-lactams function by inhibiting the transpeptidation step of bacterial cell wall synthesis by acting as a false substrate for the transpeptidase enzyme, i.e. the β-lactam is an analogue of the D-alanine-D-alanine terminal portion of the peptide side chain of the peptidoglycan MurNAc unit. This prevents cross linking (-CO-NH-inter-bridges) of the peptide chains of the glycan strands. Therefore, accumulation of peptidoglycan precursors results in activation of bacterial cell wall autolysins causing degradation of the bacteriums remaining peptidoglycan. The growing bacterium at this point is extremely susceptible to cell lysis and death.

1.2.3.1 The penicillins
The penicillin group is comprised of the four-ring β-lactam structure fused with a five-membered thiazolidine ring. This makes up the 6-aminopenicillanic acid (6-APA) molecule (Figure 1.2). The 6-APA carries a variable N-acyl side chain at position 6. Side chains are chemically added to the 6-APA molecule on the ‘R’ group of the N-acyl side chain to produce a wide variety of semi-synthetic penicillins.
Figure 1.2 General chemical Structure of the β-lactam class antimicrobials, adapted from Essack 2001 [57]

Basic chemical structure of the penicillins

Basic chemical structure of the cephalosporins

Basic chemical structure of the cephamycins

Basic chemical structure of the monobactams

Basic chemical structure of the carbapenems
The penicillin antimicrobials can be subdivided into five groups:

(A) **The natural penicillins**: The group of natural penicillins are products of fermentations of the mould *Penicillium chrysogenum* and include: pentenylpenicillin (penicillin F), benzylpenicillin (penicillin G), heptylpenicillin (K), phenoxyethylpenicillin (penicillin V), and p-hydroxybenzylpenicillin (penicillin X). These compounds differ based on the composition of the N-acyl side chain attached to the 6-APA molecule (also referred to as the ‘nucleus’). Penicillin G has activity against Gram-positive bacteria and some Gram-negatives (e.g. Meningococcal species and Gonococcal species); however, penicillin G is susceptible to hydrolysis by β-lactamase enzymes produced by many Gram-negative bacilli.

(B) **The penicillinase-resistant penicillins**: This is a group of semi-synthetic penicillins which are also referred to as anti-staphylococcal β-lactamase-stable penicillins. This group is comprised of meticillin (the first of the semi-synthetic penicillins to be discovered), nafcillin and a sub-group called the isoxazolyl penicillins (e.g. oxacillin, cloxacillin, dicloxacillin and flucloxacillin).

(C) **The extended-spectrum penicillins**: The extended-spectrum penicillins are comprised of two structurally distinct subgroups- the amino-penicillins and the amido-penicillins [58]. The first of the amino penicillins to be developed was ampicillin. Ampicillin has a similar activity as benzylpenicillin against Gram-positive cocci with an extended-spectrum of activity against certain Gram-negative organisms such as *E. coli, Salmonella* spp.

The amido-penicillins are so called as they possess an amide group at the α-position of the side chain of the benzyl penicillin molecule, and are therefore termed 6-α-amidinopenicillnates. Mecillinam is an amido-penicillin with an extended-spectrum of activity toward Gram-negative organisms, but differs from the amino-penicillins in that it has little activity against Gram-positive organisms.
Ampicillin is not effective in the treatment of infection caused by β-lactamase-producing Gram-negative bacteria. This restriction can be overcome by combining ampicillin with another compound called a β-lactamase inhibitor. This combination of β-lactam/β-lactamase inhibitor is an effective means of treating infections caused by β-lactamase-producing Gram-negative organisms.

(D) The extended-spectrum penicillins with activity against *Pseudomonas aeruginosa*:
There are three acyl-derivatives of benzylpenicillin exhibiting activity against *P. aeruginosa*. These are the carboxypenicillins (ticarcillin and carbenicillin), sulbenicillin, the acylaminopenicillin’s (apalcillin, aspoxicillin) and the acylureidopenicillin’s (azlocillin, mezlocillin, piperacillin) which all have activity against *P. aeruginosa*.

(E) β-lactamase stable penicillins: Temocillin (6-α-methoxypenicillin) was first marketed by Beecham Pharmaceuticals in the 1980’s [59]. Temocillin is a 6-α-methoxy derivative of ticarcillin which is administered parenterally. Temocillin exhibits stability against CTX-M, TEM, SHV and AmpC β-lactamases. However, Temocillin is not active against Gram positives, anaerobes or *Pseudomonas aeruginosa*.

1.2.3.2 The cephalosporins
The cephalosporin antimicrobials are slightly different in that they contain a 6-membered dihydrothiazine ring fused to the β-lactam ring rather than the thiazolidine ring (5-membered) of penicillins (Figure 1.2). The cephalosporins are all semi-synthetic derivatives of a compound called cephalosporin C that is produced by the mould *Cephalosporium acremonium*. The core cephalosporin structure is 7-aminocephalosporanic acid (7-ACA). As with 6-APA, the 7-ACA structure may be chemically altered at a number of locations to yield the different cephalosporin agents.

The cephalosporins can be classified based on biological, microbiological, pharmacological, chemical and immunological attributes. Classification may be
based upon whether the compound is administered orally or by parenteral means and also the stability of the compound to hydrolysis by β-lactamase enzymes [60]. The cephalosporins are part of the ‘cephem’ sub division of β-lactam antimicrobials. Chemical classification is based upon the structure of the cephem ring. Therefore, the cephems are sub divided into the cephalosporins (1-sulfur), 1-oxacephems, carbacephems, isocephems, cephemycins and cephabacins.

There are a number of microbiological classification schemes; one grouping the cephalosporin’s into ‘generations’ 1-5, based upon the spectrum of antibacterial activity and their stability against β-lactamase-producing Gram-negative bacteria [60]. Another accepted microbiological classification scheme distributes the various cephalosporin agents into ‘groups’ I-VII. Groups I, II cephalosporins belong to the category of limited spectrum cephalosporins, group III, IV & V to the broad spectrum cephalosporins groups VI & VII to the narrow spectrum cephalosporins [60].

➢ **Group I cephem’s:**
This cephem group possesses activity similar to ampicillin when it comes to treatment of infections caused by Gram-negative bacteria with a similar hydrolysis level by β-lactamase enzymes. Group I cephems are the most active against staphylococci and streptococci [60]. Members of this group include cephaloridine, cephalothin, cefazolin and cephadrine.

➢ **Group II cephems**
The group II cephem’s are made up of cephalosporins and cephemycins. There are two sub groups- group II-A and II-B. Group II-A agents, for example cefuroxime, are less active than the group 1 cephems against staphylococci and streptococci but more active against Gram-negative bacilli. Group II-B agents are the cephemycins.

The cephemycins (α –methoxycephalosporins) are similar in structure to the cephalosporins with the addition of a methoxy group at the C7 position of the β-lactam ring of 7-amino-cephalosporanic acid. The cephemycins include
cefoxitin, and cefotetan. Due to the 7-α-methoxy group the β-lactam ring of the cephamycins is resistant to cleavage by the Ambler Class A β-lactamase enzymes.

- **Group III cephems**
The group III cephems are diverse in their chemical structure, antimicrobial activity and pharmacokinetic properties. The group III cephems are comprised of subgroups-A to E [60]. The members possess two or more attributes:

1. Either a 2-amino-5-thiazolyl or a 5-amino-2-thiadiazolyl ring at the C7 position.
2. Broad antimicrobial spectrum of activity.
3. Stable to hydrolysis by many plasmid-mediated β-lactamase enzymes such as TEM-1.
4. Anti-pseudomonal activity.
5. MIC_{90} values of less than 1.0mg/L for members of the *Enterobacteriaceae* that do not possess Class I β-lactamase or extended-spectrum β-lactamase enzymes (refer to section 1.3).

The Group III-B cephems comprise compounds possessing a 2-amino-5-thiazolyl ring and an alkoxyimino side chain at C7. The oxime\(^5\) is substituted with either a methyl group (cefotaxime and ceftriaxone) or a carboxyisopropyl group (ceftazidime). The molecule at the C3 position in these agents differs, for example; ceftazidime possesses a pyridinium molecule, ceftriaxone a triazine molecule and cefotaxime an acetoxymethyl group at C3. The Group III-B agents are more active than the group II-A agents against the *Enterobacteriaceae* and are also stable to hydrolysis by plasmid-mediated β-lactamase enzymes. Group III-A, III-C, Group III-D and Group III-E cephems are not widely used.

**Group IV cephems**
The group IV cephems are subdivided into group’s IV-1 and IV-2 based upon the position (C3 or C7) of the quaternary ammonium moiety. Group IV-2 are the C3

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\(^5\) Oxime’s are any class of nitrogen containing compounds. They have the chemical structure X/Y/C=N-OH, where X and Y are hydrogen atoms or organic groups derived from an organic compound.
quaternary ammonium cephems including cefepime and cefpirome. Group IV cephems are active against staphylococci and Gram-negative bacteria including *P. aeruginosa*. These agents are relatively stable to hydrolysis by many plasmid-mediated β-lactamase enzymes.

At present cefepime is not authorised for human use by either the European Medicines Agency (EMEA) or the Irish Medicines Board (IMB). Cefepime is FDA approved for the treatment of moderate to severe infections such as pneumonia, uncomplicated and complicated urinary tract infections (UTI’s), skin and soft tissue infections, intra-abdominal infections and febrile neutropenia [61].

Cefepime is a zwitterionic⁶ oxymino β-lactam with an amino-thiazole side chain. In contrast to the earlier cephalosporins, cefepime penetrates the bacterial cell more rapidly. The major mechanism of resistance to cefepime in Gram-negative bacteria is hydrolysis by β-lactamase enzymes although cefepime escapes the effects of many chromosomal and plasmid-mediated β-lactamase enzymes due to their low affinity for this cephalosporin [62]. The cefepime molecule possesses a methylpyrrolidinium group which confers a zwitterionic charge. The zwitterionic charge is bactericidal in that it ensures rapid penetration of the methylpyrrolidinium group through the outer membrane porins of Gram negative bacteria.

The group V, VI and VII cephems are not directly relevant to this thesis and will not be discussed.

1.2.3.3 The monobactams and carbapenems

The monobactams are a group of β-lactam agents which are monocyclic (Figure 1.2). This is in contrast to other β-lactam agents which are fused to a secondary ring structure. The monobactams are natural products of bacteria not fungi or actinomycetes. Naturally occurring monobactams exhibit poor antimicrobial activity. However, modification of the monocyclic monobactam results in a

⁶ A zwitterionic molecule is a molecule that carries both a positive and negative charge.
potent antibacterial agent. This modification is achieved by the addition of an aminothiazole-oxime side chain to position C3 and the addition of an α-methoxy group to position C4 of the monobactam β-lactam ring. This α-methoxy group protects the monobactam β-lactam ring from cleavage by β-lactamase enzymes. Members of this group of β-lactam include aztreonam. Aztreonam is active mainly against aerobic and facultatively anaerobic Gram-negative bacteria.

The carbapenems are the most recently developed sub-class of β-lactam agents and exhibit the broadest spectrum of antibacterial activity. These agents have been isolated from the fermentation products of a variety of Streptomyces. The most commonly administered members of the carbapenems include meropenem, imipenem, doripenem and ertapenem. The carbapenems differ from the penicillins by a carbon atom in place of a sulphur atom in the five membered ring and a double bond between C2 and C3 (Figure 1.2). These agents have a hydroxyl-ethyl group at the C6 position which is in contrast to the aminoacyl group found at this position in the majority of the other β-lactam agents. The carbapenem’s exhibit antimicrobial activity against a very broad range of Gram-positive and Gram-negative aerobes and anaerobes. In the past, these agents have been stable to most β-lactamase enzymes; however, carbapenemases that are capable of hydrolysing carbapenem agents are becoming increasingly important.

1.2.3.4 β-lactamase inhibitors- a focus on clavulanic acid

One of the main mechanisms of resistance to β-lactam antimicrobials is the production of β-lactamase enzymes. A combination of a β-lactam agent such as amoxicillin or ampicillin with a β-lactam agent that is capable of inhibiting the β-lactamase enzyme can overcome this resistance. The most commonly utilised β-lactamase inhibitors are clavulanic acid and the penicillanic acid sulphones-sulbactam and tazobactam.

Clavulanic acid was discovered by Brown and colleagues in 1976 as a product of the bacteria Streptomyces clavuligerus. In relation to its chemical structure, clavulanic acid is an analog of the basic β-lactam structure, with an oxygen atom substituted for a sulphur atom, and therefore an oxazolidine ring structure in
place of a thiazolidine ring. This similarity in chemical structure enables clavulanic acid to act as an inhibitor of β-lactamase activity.

Clavulanic acid has low intrinsic activity against most Gram-positive and Gram-negative bacteria and therefore clavulanic acid itself is not useful as a sole therapeutic agent. Instead, clavulanic acid is formulated with a broad-spectrum β-lactam agent e.g. amoxicillin, ticarcillin, piperacillin. This partner β-lactam agent is susceptible to β-lactamase enzymes. Clavulanic acid binds and inhibits β-lactamase that would otherwise inactivate the partner β-lactam e.g. amoxicillin, resulting in amoxicillin having an expanded spectrum of activity. This activity is possible as clavulanic acid acts as an irreversible inhibitor of β-lactamase enzymes that degrade and inactivate β-lactam antimicrobials.

Clavulanic acid was the first clinically useful β-lactamase inhibitor to be described and is an irreversible ‘suicide’ inhibitor (a compound which resembles the normal substrate for an enzyme, but which interacts with the enzyme to form a covalent bond and thus inactivates the enzyme). For a β-lactamase inhibitor to be successful clinically it needs to have not only intrinsic activity against a β-lactamase enzyme but also be capable of readily penetrating into the bacterial cell periplasm [63]. Farmer and colleagues illustrated that penetration into the periplasm by clavulanic acid was far superior to that of other β-lactamase inhibitors such as sulbactam, tazobactam and BL42715 (a penem inhibitor) [63].

1.3 β-lactamase and extended-spectrum β-lactamase (ESBL) enzymes

There are a variety of mechanisms of resistance to commonly used antimicrobials such as the β-lactam’s [64] [65]:

- Enzyme production causes degradation and/or modification of an antimicrobial e.g. β-lactamase.
- Efflux pumps result in the rapid expulsion of an antimicrobial from the cell, e.g. MexAB-OprM efflux pump system in *P. aeruginosa* and AcrAB ToIC efflux pump system in *E. coli*. 
• Existence of modified transpeptidases that are not susceptible/less susceptible to inhibition by β-lactams e.g. penicillin binding proteins (PBP’s).
• Impermeability of the outer membrane to antimicrobials, e.g. outer membrane porin loss so that the antimicrobial is unable to penetrate into the periplasmic space

1.3.1 Classification/ nomenclature of β-lactamase enzymes
β-lactamase enzymes are the predominant method of resistance to β-lactam antimicrobial agents among the Enterobacteriaceae. A website set up in 2001 by Karen Bush and George Jacoby at the Lahey Clinic is dedicated to tracking and recording all new β-lactamases discovered [66]. The two most commonly used classification schemes for the β-lactamase enzymes are the Ambler scheme and the Bush-Jacoby-Medeiros scheme (BJM) [67]. The Ambler scheme classifies β-lactamases based on their protein homology whereas the BJM scheme classifies β-lactamases based on their substrate and inhibitor profiles.

- **The Ambler classification scheme**
The Ambler Class A, C and D β-lactamases are termed serine β-lactamases as they possess an active site serine residue to bind to the β-lactam ring. The Class B β-lactamases are termed zinc metallo enzymes as they possess active site zinc ions.

- **BJM scheme 1995 and updated Bush Jacoby scheme 2010**
There has been much debate as to the exact definition of an ESBL. According to the BJM 1995 scheme an ESBL is defined as ‘a functional class 2be, molecular class A, clavulanate inhibited β-lactamase which can hydrolyse oxyiminocephalosporins at rates at least 10% of that for benzylpenicillin’ [68] [67]. However, this definition excluded all other β-lactamase enzymes with an extended-spectrum of hydrolysis (clavulanic acid inhibitable or otherwise) such as the OXA β-lactamases, plasmid-mediated AmpC enzymes, OXA-carbapenemases, Class A carbapenemases and the Class B metallo-β-lactamases (MBL’s). Therefore, every β-lactamase that was not in the BJM Group 2be (1995
classification)/ Ambler class A was excluded from the definition. However, many of these excluded enzymes are capable of hydrolysing the extended-spectrum β-lactam antimicrobials. Recognising the need to correlate ESBL classification with the phenotype observed in clinical isolates, Bush and Jacoby have recently published an update to their 1995 classification scheme [69]. This scheme designates a number of new sub groups based on the increasing knowledge in the field of ESBL’s over the past 15 years. The new sub groups proposed are Groups 1e, 2ber, 2ce, 2de and 2df. The hydrolysis profile and inhibition characteristics of the updated Bush Jacoby 2010 groupings are outlined in Table 1.2.

- **Giske and colleagues classification scheme**

Identifying a need for a more comprehensible and accessible nomenclature for β-lactamase enzymes in the clinical setting and policy domain, Giske and colleagues put forward a revised nomenclature for ESBL enzymes [68].

This revised nomenclature divides ESBLs into three main groups- ESBL\textsubscript{A}, ESBL\textsubscript{M} and ESBL\textsubscript{CARBA}. All β-lactamase enzymes of Ambler Class A/BJM Group 2be are represented by the term Class A ESBL\textsubscript{S} or ESBL\textsubscript{A}. OXA-ESBL\textsubscript{S} and plasmid-mediated AmpC’s are to be termed ‘miscellaneous’ ESBL’s or ESBL\textsubscript{M}. The carbapenem-hydrolysing β-lactamase enzymes are represented under the designation ESBL\textsubscript{CARBA}. The following operational definitions are used:

- **ESBL\textsubscript{A}** - ‘non-susceptibility to extended-spectrum cephalosporins (ESC’s) and clavulanate synergy’
- **ESBL\textsubscript{M}** - ‘non-susceptibility to ESC’s and phenotypic detection (ESBL\textsubscript{M-C}) or genotypic detection (ESBL\textsubscript{M-D})’
- **ESBL\textsubscript{CARBA}** - ‘non-susceptibility to ESC’s and at least one carbapenem and ESBL\textsubscript{CARBA} detected with phenotypic and/or genotypic methods’

There are further subdivisions within each of the main groupings for example ESBL\textsubscript{M} is divided into ESBL\textsubscript{M-D} and ESBL\textsubscript{M-C}. ESBL\textsubscript{M-D} represents those miscellaneous ESBL’s belonging to Ambler Class D. ESBL\textsubscript{M-C} represents
plasmid-mediated ESBL’s belonging to the Ambler Class C group of β-lactamases.

For the purpose of clarification of its use throughout this thesis, the term ‘ESBL’ will be used to discuss enzymes that confer resistance to one or more of the 3rd or 4th generation cephalosporins and are inhibited by clavulanic acid. These enzymes will fall into Ambler class A & D, Bush Jacoby 2010 groups 2be, 2ce, 2de & 2e and Giske groups ESBL_A & ESBL_M.
Table 1.2 Updated classifications of β-lactamase enzymes, data sourced from Bush and Jacoby 2010, Giske et al. 2009 [68, 69]

<table>
<thead>
<tr>
<th>Ambler</th>
<th>Giske et al. 2009</th>
<th>BJM 1995</th>
<th>BJ 2009</th>
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<th>Inhibited by</th>
<th>Defining characteristics</th>
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<td></td>
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<td>1</td>
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<td>No</td>
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<td>2b</td>
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</table>

BJM= Bush, Jacoby and Medeiros, BJ= Bush and Jacoby, CA= clavulanic acid, TZB=tazobactam, EDTA= ethylenediaminetetraacetic acid
NI= not included in this scheme, ES= Extended-spectrum
1.3.2 Structure of β-lactamase enzymes
Each Ambler class of β-lactamase contains a particular ‘signature’ sequence or motif at the active site [70]. The three dimensional enzymatic structure of β-lactamases has not been studied extensively, with Class A being investigated the most to date. However, the exact catalytic mechanisms of these structures are not fully understood and so far the functions of many of the catalytic residues are only hypothesized [71-73].

1.3.3 Ambler class A β-lactamases
- **TEM-type β-lactamases**
The first plasmid-mediated β-lactamase was discovered in *E. coli* in the 1960’s. The enzyme was given the designation TEM-1 after the patient from which the bacterium harbouring the enzyme was isolated- *Temoniera* [74]. The original TEM-type β-lactamases (TEM-1, TEM-2 and TEM-13) confer resistance to the penicillins as a group, the narrow-spectrum cephalosporins (e.g. cephalothin) and are susceptible to β-lactam inhibitors such as clavulanic acid. The TEM enzymes are most frequently reported in *E. coli* and *Klebsiella* spp. but with with increasing reports of detection in other *Enterobacteriaceae* (e.g. *Salmonella enterica* and *Enterobacter* species). TEM-1 is also frequently found in *Haemophilus influenzae*. TEM-1 and its derivative TEM-2 possess the same substrate hydrolysis profile; however, the two enzymes differ at position number 39 with a single amino acid substitution of lysine for glutamic acid. TEM-13 is the other TEM-type β-lactamase observed with a narrow spectrum substrate profile [75]. These enzymes belong to the Ambler class A, BJM group 2b classifications.

- **TEM-ESBLs**
There are now more than 180 TEM-type β-lactamase enzymes discovered to date with isoelectric points (pI’s) ranging from 5.2-6.5 [66] [76]. One or more amino acid substitutions in the TEM enzyme sequence causes alterations of the substrate profile and result in hydrolysis of the expanded spectrum cephalosporins. The TEM-ESBL enzymes retain the ability to hydrolyse the penicillins; however, to a much lesser degree than the parent TEM enzyme [77].
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The first TEM-type ESBL was reported in the late 1980’s [78]. This enzyme was originally designated CTX-1 due to the enzyme’s ability to hydrolyse cefotaxime. However, shortly afterwards the enzyme was renamed TEM-3. A range of amino acid changes that associated with the ability to hydrolyse the oxyimino- cephalosporins have been described. It is important to point out that a number of TEM-ESBLs have evolved from TEM-2 and therefore harbour the lysine-glutamine amino acid substitution at position number 39, with additional amino acid substitutions enabling hydrolysis of oxyimino- cephalosporins. The extended-spectrum TEM enzymes also confer resistance to aztreonam and are susceptible to β-lactamase inhibitors such as clavulanic acid. The TEM type ESBL’s belong to Ambler’s molecular class A, BJM group2be.

➢ Other TEM variants

In addition to the TEM-type β-lactamase’s and TEM-type ESBL’s, there are two other clinically-relevant subgroups of the TEM enzymes; the inhibitor-resistant TEM (IRT) enzymes and complex mutant TEM (CMT) enzymes. Members of the IRT sub group confer resistance to the penicillins, are susceptible to cephalosporins; however, importantly they are less susceptible to β-lactam/ β-lactamase inhibitor combinations [79]. IRT’s are Ambler Class A, Bush Jacoby group 2br β-lactamases.

The CMT enzymes possess the combined properties of both IRT’s and ESBL’s [77]. These enzymes were shown to confer resistance to the oxyimino cephalosporins (at a lesser level than the TEM-ESBLs) and to β-lactamase inhibitors with hydrolysis of penicillins being less efficient than that of the TEM-1 enzyme [80]. The level of substrate hydrolysis depends on the particular mutations encountered. Amino acid substitutions at two to five positions in the TEM-1 enzyme account for the CMT enzyme variants. There are 10 CMT variants identified to date [66]. CMT’s are Ambler Class A, Bush Jacoby group 2ber β-lactamases.
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➢ **SHV-type β-lactamase**

The designation ‘SHV’ refers to the term ‘Sulphhydryl Variable’. This term was employed to describe a biochemical property of the enzyme now known as ‘SHV’. SHV-1 is present on the chromosome of *K. pneumoniae* and reportedly accounts for 20% of ampicillin resistance in this species [77]. The enzyme was originally discovered by Pitton in 1972 and was termed Pit-2 in 1979 by Matthew and colleagues [81]. TEM-1 and SHV-1 enzymes share 68% sequence identity [82]. Mobilization of the *bla*<sub>SHV</sub> gene from the chromosome of *Klebsiella* species has been associated with the rapid dissemination of the enzyme to other members of the *Enterobacteriaceae* such as *E. coli*, *Enterobacter* spp. and other non-*Enterobacteriaceae*—*P. aeruginosa* and *Acinetobacter* spp. [76, 77, 83, 84]. There are more than 130 SHV-type enzymes discovered to date with pI’s ranging from 7.0-8.2 [66]. The first SHV-type ESBL, SHV-2, was reported in Germany in 1983 in *Klebsiella ozaenae* [76]. SHV ESBLs belong to Ambler Class A, Bush Jacoby group 2be.

➢ **Other SHV variants**

In addition, like the TEM group of ESBL’s there are rare incidences of inhibitor-resistant variants of the SHV enzymes (IRS). To date there are three IRS enzymes identified—SHV-10, SHV-49 and SHV-56. All three IRS’s have been identified in clinical *K. pneumoniae* isolates [85-87]. The hydrolysis profile exhibited by IRS enzymes is similar to that of IRT enzymes. These enzymes belong to Ambler Class A, Bush Jacoby group 2br.

➢ **CTX-M type ESBLs**

The designation ‘CTX-M’ refers to ‘cefotaximase’. These enzymes share c.40% sequence homology with TEM and SHV β-lactamase [77]. Cefotaximase is a term relating to the preferential hydrolytic activity of this group of enzymes for cefotaxime (MIC’s >64 μg/ml) compared to ceftazidime. The level of ceftazidime hydrolysis by CTX-M enzymes generally does not result in high level ceftazidime resistance in clinical isolates (MIC’s 2-8μg/ml). It is however important to note that certain CTX-M enzymes have the ability to hydrolyse ceftazidime at varying rates. For example, ceftazidime resistance (at levels up to
256μg/ml) can be conferred by the CTX-M-15 & -19 enzymes [88]. In addition, certain CTX-M enzymes have the capacity to hydrolyze cefepime at levels (MIC’s 4 - ≥64μg/ml) that confer resistance [89]. The CTX-M family of β-lactamases are susceptible to inhibition by β-lactam inhibitors.

The first CTX-M type ESBL’s were detected independently in Germany in 1990, in France in 1987 and in Japan in 1988 [90]. Originally the enzyme was termed MEN-1 (E. coli strain MEN). CTX-M genes are plasmid-encoded resistance determinants and are believed to have arisen due to mobilisation of a chromosomal β-lactamase gene from the Kluyvera species. There are five sub-groups of the CTX-M family based on amino acid sequence similarities: CTX-M Group 1, Group 2, Group 8, Group 9 and Group 25.

The Group 1 enzymes are structurally related to a β-lactamase naturally occurring in Kluyvera cryocrescens, Group 2 to K. ascorbata, Group 8 and 25 to K. georgiana, with Group 9 CTX-M’s shown to be similar to a β-lactamase isolated from a Kluyvera spp in Guyana [88, 91]. At present there are over 100 CTX-M-type enzymes identified with pIs ranging from 7.6-9.0 [66]. CTX-M ESBL’s are most often found in E. coli with CTX-M variants also identified in other members of the Enterobacteriaceae with frequent identification occurring in Klebsiella, Serratia, Enterobacter and Salmonella species.

1.3.4 Ambler Class B β-lactamases

Class B β-lactamases or metallo β-lactamases differ from the Class A, C and D serine β-lactamases in that they require zinc ions to catalyze hydrolysis. Class B β-lactamases can be sub-classed in to B1, B2 and B3. Sub-class B1 and B3 are broad spectrum in that they hydrolyze most β-lactam antimicrobials, the carbapenems and are resistant to serine β-lactamase inhibitors. Sub-class B2 are strict carbapenemase enzymes and do not effectively hydrolyze the penicillins nor the cephalosporins. The functional characteristics distinguish each sub-class.

Sub-class B1 comprises both chromosomal and plasmid-mediated variants. Sub-class B2 and B3 are chromosomally encoded. BcI was the first Class B β-
lactamase identified in 1966 and this was chromosomally encoded in *Bacillus cereus* [92]. Additional chromosomal sub-class B1 β-lactamases are BlaB (isolated from *E. meningoseptica*) and CcrA (isolated from *Bacillus fragilis*). The plasmid-mediated variants of Class B β-lactamases include IMP-variants, VIM-variants, SIM-variants, SPM-variants and GIM-variants. In 1994 the first plasmid-mediated Class B β-lactamases was identified in *P. aeruginosa* [92]. In addition the presence of Class B β-lactamases in the environment highlight’s environmental bacteria, as a reservoir for transmission of Class B β-lactamases into bacteria of clinical significance.

Most recently, a Class B β-lactamase termed NDM-1 (New Delhi β-lactamase) has become a cause for concern. The gene was isolated from a *Klebsiella pneumoniae* isolate from a Swedish patient that was admitted previously to an Indian hospital [93]. This gene confers resistance to the all β-lactam antimicrobials and is generally carried on plasmids conferring resistance to other classes of antimicrobial agents such as the fluoroquinolones and the aminoglycosides. This gene has recently been reported in other countries such as the UK, Pakistan and India [94].

### 1.3.5 Ambler Class C β-lactamases

The first enzyme identified that was capable of destroying penicillin was encoded by the *ampC* gene. This gene was located on the chromosome of an *E. coli* strain isolated in 1940 [95]. However, the actual sequence of this gene was not determined until 1981. The results showed that the *ampC* gene was similar to the TEM-1 β-lactamase in possessing an active site serine residue. Other organisms known to possess an *ampC* gene on their chromosome include *Citrobacter* spp., *Enterobacter* spp., *Shigella* spp., *Yersinia* spp., *Acinetobacter* spp. and *Pseudomonas* spp. Organisms may possess either an inducible or non-inducible chromosomal *ampC* gene. An inducible *ampC* gene is one that is up-regulated upon exposure to a β-lactam agent. *P. aeruginosa*, *C. freundii*, *E. cloaceae* and *S. marcesans* possess an inducible *ampC* gene that may be expressed at high levels in the presence of a β-lactam agent [96]. *E. coli*, *Acinetobacter baumannii*, and *Shigella* possess a non-inducible *ampC* gene. In
organisms with chromosomal ampC (inducible or non inducible) mutations can result in sustained high level expression which is clinically significant.

AmpC enzymes are capable of hydrolysing the cephalosporins and the cephemycins (e.g. cefoxitin). These enzymes are not inhibited by clavulanic acid, tazobactam or sulbactam. AmpC enzymes are however inhibited by oxacillin, cloxacillin, cefepime and aztreonam. There are currently 107 AmpC enzymes identified with pI’s generally being greater than 8.0 [66]. The conserved catalytic residues in ampC enzymes are serine at position 64, lysine at position 67, tyrosine at position 150, asparagine at position 152, lysine at position 315 and alanine at position 318. Amino acid substitutions at these sites reduce the catalytic efficiency of the AmpC enzyme [97].

Plasmid-mediated ampC genes have originated from chromosomal ampC genes. The plasmid mediated ampCs are derived from the chromosomal ampC’s of members of the Enterobacteriaceae including E. cloaceae, Citrobacter freundii and Morganella morganii. Plasmid-mediated ampCs exhibit a high degree of sequence homology with chromosomal ampC genes with minor difference in sequence homology giving rise to the various plasmid-mediated AmpC families. The plasmid-mediated ampC genes are found in both nosocomial and non-nosocomial bacterial isolates with the genes being commonly found in Enterobacteriaceae that do not possess an intrinsic AmpC.

In relation to the first report in the literature on plasmid-mediated ampC, a number of reports are documented. In 1976 Bobrowski and colleagues reported a plasmid-mediated ampC in Proteus mirabilis that was indistinguishable from the chromosomal ampC of E. coli. However, the plasmid was lost from the P. mirabilis isolate before characterization on the enzyme was complete [98]. In 1982 Levesque and colleagues reported a plasmid-mediated cephalosporinase in Achromobacter species, however- again- characterization on the enzyme was not completed as the bacterial isolate was lost [99]. In 1989 Baureinfeind and colleagues identified the first plasmid-mediated ampC gene (CMY-1) in Klebsiella pneumoniae isolated from a patient in South Korea [100]. Plasmid-
mediated \textit{ampC} s are most often found in \textit{Enterobacteriaceae} isolates that do not possess a chromosomal \textit{ampC} or, as in the case of \textit{E. coli}, with a non-inducible \textit{ampC}. CMY is the most common plasmid-mediated \textit{ampC} identified. There are 64 plasmid-mediated variants of CMY [66]. Other plasmid-mediated \textit{ampC}’s include DHA variants, ACC variants, MOX variants, FOX variants, LAT variants, BIL-1, MIR-1 and ACT-1.

1.3.5 Ambler Class D \( \beta \)-lactamases

Class D \( \beta \)-lactamases, also referred to as ‘oxacillinases’, are serine \( \beta \)-lactamases designated by the acronym ‘OXA’. The OXA enzymes preferentially hydrolyze oxacillin and cloxacillin compared to benzylpenicillin. OXA enzymes are unique due to their inhibition by sodium chloride (NaCl) [101].

The Class D \( \beta \)-lactamases are the most diverse of the four Ambler molecular classes, with identities ranging from 16%-99% between individual member enzymes [102]. Class D enzymes exhibit about 16% sequence identity with the Class A enzymes [103]. There are over 190 OXA -type enzymes identified to date with pI’s ranging from 5.5-8.1 [66]. The hydrolysis profiles of class D members range from narrow-spectrum \( \beta \)-lactams, to extended-spectrum \( \beta \)-lactams and to the carbapenems.

It is a key point in relation to the work presented in this thesis that the literature indicates that members of Class D are generally not inhibited by clavulanic acid, EDTA, tazobactam or sulbactam [104, 105]. Exceptions to this are OXA-9, OXA-18, OXA-45 and OXA-53 [104].

Genes encoding OXA enzymes can be found located on the chromosome with chromosomal OXA genes found exclusively in the \( \gamma \) sub-division of Gram-negative proteobacteria. The first organism identified to possess an intrinsic occurring OXA gene was \textit{Aeromonas jandeai} (formerly \textit{A. sobria}) and this was the OXA-12 gene [106].
Unlike class A, B and C β-lactamases, homo-dimeric forms\(^7\) have been reported for class D β-lactamases e.g. OXA 10 & 14 [101, 107-109]. Of all the β-lactamase classes, class D β-lactamase enzymes are probably the least studied and understood [110] [101]. A phylogenetic study carried out by Barlow and Hall in 2002 illustrated that an ancestral OXA homologue originated in Gram-positive bacteria with the transition into Gram-negative occurring approximately 575-520 million years ago [111]. Chromosomal OXA genes in Gram-negative bacteria have been mobilized to plasmids at least 3 different times over a period of c.150 million years [111]. The first mobilisation event was estimated to have occurred c. 116 million years ago, the second c. 42 million years ago with the third mobilisation of OXA not able to be estimated accurately. Members that arose during the first and second mobilisation events include OXA 3 and OXA-5. OXA’s resulting from recent mobilisation events (time not estimated) include group III (OXA-1, 31), group II (OXA-2, 15, 34 & 32) and group I (OXA-10, 19, 28 & 35).

The acquired narrow-spectrum class D (NSCD) β-lactamases can be sub-divided into [112]:

- **Sub group I (OXA-10- Like)** - Including OXA-5, 7, 10, 11, 13, 14, 16, 17, 19, 28, 35, 48, 54, 55, 56 & 101
- **Sub group II (OXA-2- Like)** - Including OXA-2, 3, 20, 21, 34, 36, 37 & 46
- **Sub group III (OXA-1- Like)** - OXA-1/30, 4, 31, 33 & 47
- **Sub group IV** - OXA-9, 12, 22, 29, 42, 43, 57, 59, AmpS and LoxA
- **LCR-1 Sub group V** - LCR-1 only, grouped alone as very little homology between LCR-1 and other class D members; LCR-1 is weakly related to OXA-53 (c.40%). The ‘LCR’ acronym represents the initials of the patient from which the bacterium (*P. aeruginosa*) harbouring the β-lactamase was isolated from- L.C. The ‘R’ standing for ‘resistant’ [113].

\(^7\) Homo-dimeric enzymes are formed by the combination of two identical monomers, each monomer having a separate active site. The dimeric form is more active kinetically than the monomeric form of the enzyme and is formed in solution, at high concentrations (e.g. in stock solutions).
The acquired extended-spectrum class D (ESCD) β-lactamases can be classified as:

- Point mutations of narrow-spectrum class D β-lactamases- of sub-group II, OXA-2-like (this includes OXA-15 & OXA-32 which have an extended-spectrum of hydrolysis compared to parent enzyme OXA-2, however, are they are not inhibited by clavulanic acid).

- Extended-spectrum class D β-lactamases (ESBLs) not structurally related to narrow-spectrum class D β-lactamases-(this includes OXA-18, 45 & 53 which are inhibited by clavulanic acid (OXA-ESBLs).

➤ **OXA’s inhibited by clavulanic acid-‘OXA-ESBLs’**

OXA-18 was the first OXA β-lactamase identified which was also significantly inhibited by clavulanic acid (the first OXA-ESBL). The gene was isolated from the chromosome of a clinical *P. aeruginosa* strain [114, 115]. The ceftazidime, cefotaxime and cefepime MIC’s for OXA-18 producing *P. aeruginosa* was reported as 128µg/ml, 128µg/ml and 16µg/ml and these were reduced to 8µg/ml, 8µg/ml and 4µg/ml when each cephalosporin was combined with clavulanic acid [114].

OXA-45 was identified on a 24kb plasmid in a multi-drug resistant clinical *P. aeruginosa* strain from Texas, USA [102]. OXA-45 shares 65.9% sequence homology with OXA-18 [102]. The ceftazidime, cefotaxime and cefepime MIC’s for OXA-45 producing *E. coli* was reported as 128µg/ml, 4µg/ml and 4µg/ml, which were reduced to 0.12µg/ml, 0.03µg/ml and 0.03µg/ml when each cephalosporin was combined with clavulanic acid [102].

Most recently clavulanic acid-inhibitable OXA-53 has been identified on a plasmid in a *S. Agona* isolate from Brazil [116]. The ceftazidime, MIC for OXA-53 producing *S. Agona* was reported as >32µg/ml, which was reduced to 1.5µg/ml, when the cephalosporin was combined with clavulanic acid [116].
Class D carbapenemases - OXA carbapenemases

Carbapenem-hydrolysing variants of OXA represent a sub group of enzymes which confer the capability of hydrolysing essentially all β-lactam antimicrobials. There are 9 sub-groups of OXA carbapenemases (class D carbapenemases) which have been established based upon amino acid homologies. The first OXA with carbapenemase activity (ARI-1 now called OXA-23) was identified in 1993 from a multidrug resistant A. baumannii isolate from Scotland [105].

Carbapenemases are a growing public health problem at this time however they are not central to this thesis and will not be considered further.

1.3.5.1 A focus on OXA-1 β-lactamase

OXA-1 is also referred to as OXA-30 in some publications. The reason for this is that there was a sequencing error in the original OXA-1 amino acid sequence deposited in GenBank [117]. The original OXA-1 sequence was documented in GenBank to possess an arginine residue at position 128. The OXA-30 sequence that was deposited into GenBank upon its discovery contained a glycine at position 128. However, Sun et al confirmed that OXA-1 actually possessed an arginine residue at position 128 [101]. Therefore, OXA-1 is the same enzyme as OXA-30. Hereafter, ‘OXA-1’ will be used to discuss this enzyme.

OXA-1 was originally identified in E. coli and thereafter has been identified in P. aeruginosa, in Shigella spp., and in epidemic strains of Salmonella [101, 104]. The mobile genetic element associated with OXA-1 is the Tn2603 transposon with OXA-1 generally inserted into a class 1 integron [104]. Various studies have reported bla_{OXA-1} in conjunction with additional β-lactamase genes e.g. bla_{OXA-1} + bla_{TEM-1}, bla_{OXA-1} + bla_{PSE-1}, bla_{OXA-1} + bla_{PSE-1} + bla_{TEM-1} [118, 119] bla_{OXA-1} + bla_{CTX-M-15} + bla_{TEM-1} [120], bla_{OXA-1} + bla_{CTX-M-15} [121] and bla_{OXA-1} + bla_{CTX-M-37} + bla_{TEM-63} [122].

OXA-1 is capable of hydrolysing amino and ureido penicillins and certain cephalosporins. The published OXA-1 hydrolysis profiles includes resistance to ampicillin, β-lactam/ β-lactam inhibitor combinations (piperacillin/ piperacillin-
tazobactam, amoxicillin/ amoxicillin-clavulanic acid) and reduced susceptibility to cefepime [123-129]. OXA-1 is not capable of hydrolysing ceftazidime [104]. High-level resistance to cefepime as a result of OXA-1 is observed in P. aeruginosa. There have been sporadic reports of high cefepime MIC’s resulting from OXA-1 expression in clinical E. coli strains from Singapore (256µg/ml) [130]. In this study the effect of clavulanic acid inhibition when combined with cefepime was not investigated. In addition, OXA-1 expression is reported to increase the cefepime MIC for S. Typhimurium (2-16µg/ml) [131]. Again, in this study the effect of clavulanic acid inhibition when combined with cefepime was not reported.

Factors that may influence the observed β-lactam MIC may include insertion elements acting as additional promoters for blaOXA-1, hyperproduction of efflux pumps (e.g. MexAB-OprM in P. aeruginosa [132] and alteration in outer membrane porin channels, e.g. loss of Omp-F [133-135]. Despite these factors, cefepime is recommended for empiric treatment of OXA-harbouring Enterobacteriaceae and P. aeruginosa in the USA [136]. Cefepime is not licensed for use in Europe (www.imib.ie , www.ema.europa.eu).

All reports of OXA-1 substrate hydrolysis profile indicate that the enzyme is not inhibited by clavulanic acid [72, 77, 101, 104, 110]. Recent work has identified inhibition of the OXA-1 enzyme with sodium chloride, acyl phosphates, phosphonates, penicilllnates and novel penem inhibitors [72, 101, 110].

1.4 Treatment and clinical significance of β—lactamase-producing bacteria

There are a number of predictors that may be considered when it comes to the clinical outcome of infections caused by ESBL-producing bacteria. These predictors were recently discussed in a study carried out by Marchaim et al. [137]. These factors included 1) Patient factors, e.g. -age of the patient, coexistence of chronic conditions, 2) Hospital related factors, e.g. - recent hospitalisation or admission from a long term care facility (i.e. nursing home) 3) Treatment related factors, e.g. - recent surgery, recent antimicrobial therapy and
4) Severity of condition upon admission to the hospital, e.g. severe sepsis or multi-organ failure. Adverse outcomes vary from a delay in administration of appropriate treatment to an increase in mortality rates with an increase in associated cost of care also being a cause for concern [137]. Young Lee and colleagues documented increase in hospital costs and length of stay per patient infected with an ESBL-producing *E. coli* or *Klebsiella* species when compared to a patient with an infection from an organism other than an ESBL producer. The costs were $16,451 higher and the length of stay 21 days longer for an ESBL-positive patient compared to an ESBL-negative patient [138]. In addition, the clinical failure rate for patients infected with an ESBL-producing *E. coli* or *Klebsiella* spp. was 52% compared to the control population of 14% [138].

The treatment options for ESBL-producing bacteria include the carbapenems, piperacillin-tazobactam, the aminoglycosides, fluoroquinolones, tigecycline, cefepime, temocillin, colistin, nitrofurantoin and polymyxin B [139]. The antimicrobials commonly used to treat ESBL-producing bacteria in complicated UTIs and a list of their advantages and disadvantages are outlined in a recent paper [140]. Co-existence of ESBL genes on mobile genetic elements that also harbour antimicrobial resistance genes for the fluoroquinolones and the aminoglycosides is widely documented. This results in a decrease in the available options for antimicrobial therapy if a patient possesses an organism that harbours a plasmid containing multiple antimicrobial resistance genes.

### 1.5 Epidemiology and dissemination of β-lactamase enzymes

#### Class A

In recent years the types of ESBL’s reported has shifted from TEM and SHV derivatives towards the CTX-M group of ESBL’s [141] and it has been stated that only in North America are TEM and SHV variants still dominant [142-144]. The class A ESBL’s most commonly identified in Europe include- CTX-M-1, 2, 3, 9, 14, 15 & 32, TEM-24 & 52 and SHV-5 & 12 [141]. The prevalence of ESBL-producing isolates varies among countries. It was reported that 34% of *Klebsiella* species in intensive care units in Portugal carry an ESBL gene compared to only 3% in Sweden [76]. ESBL-producing isolates are also
prevalent in the clinical setting in North America, Australia, Asia, Africa and the Middle East. It has been suggested that CTX-M-type ESBL’s may account for up to 50% of ESBL-positive E. coli strains in European countries [145]. CTX-M Group 1 enzymes are the most commonly encountered ESBLs in most of Europe with CTX-M group 9 frequently identified in Spain [144, 146].

Most TEM, SHV and CTX-M ESBLs are carried on plasmids that also carry resistance genes targeted towards other classes of antimicrobials. TEM and SHV producers generally exhibit cross resistance to aminoglycosides, tetracyclines and sulphonamides. The majority of CTX-M producers also carry plasmid-mediated genes aiding in resistance to the fluoroquinolones (qnr variants,aac-6'-lb-cr genes, qepA gene) [143, 146]. The emergence of CTX-M variants in Western Europe is of increasing concern, especially the rapid dissemination in the community setting. In a recent study carried out by Morris and colleagues, the most common ESBLs identified in the community in Ireland are CTX-M-Group 1 and CTX-M-Group 9 [147]. The blaTEM variant was present in 56% and blaSHV in 18% of CTX-M producing Enterobacteriaceae analysed in this study [147].

➢ Class C
In the USA the most common plasmid-mediated ampC are CMY-2, ACT-1, DHA-1 and FOX-5, in Korea CMY-1, CMY-10-like, CMY-18-like and DHA-1, in Greece LAT-3 and LAT-4, in the United Kingdom CIT-like, CMY-2, CMY-7, CMY-21, CMY-23, ACC-like, FOX-like and DHA-like and in Canada CMY-2 predominates [97].

➢ Class D
Epidemiological data on the geographical spread of OXA β-lactamases is lacking, perhaps due to the difficulty in their identification in the clinical laboratory. Many of the OXA β-lactamases have only been reported on one or two occasions. The OXA β-lactamases have predominantly originated in France and Turkey [76, 77]. The number and clinical significance of OXA β-lactamases
is becoming better recognised, especially as their host range broadens from \textit{Pseudomonas} spp. and \textit{Acinetobacter} spp to members of the \textit{Enterobacteriaceae}.

1.6 \textbf{β-lactamase-mediated antimicrobial resistance in Salmonella}

1.6.1 \textbf{β-lactamases and \textit{Salmonella} of human origin}

The first report of Salmonella resistant to the extended-spectrum cephalosporins was in 1988 in Tunisia. The β-lactamase identified was SHV-2 and it was harboured by a \textit{Salmonella} Wien isolate from a neonatal care unit [148]. Arlet and colleagues in 2006 carried out a review of the published reports on the prevalence of Salmonella resistant to extended-spectrum cephalosporins from different countries. This group found that the prevalence of Salmonella resistant to extended-spectrum cephalosporins ranged from 0-3.4% between continents and this progressively increased year on year from 0.1% in 1996 to 1.9% in 1999 in the USA and from 0.4% in 1999 to 1.5% in 2003 in Taiwan [149]. A similar trend was observed in Belgium with the highest prevalence recorded in 2004 at 3.4% [149]. In 2004, forty-three countries had reported having a public health problem with extended-spectrum cephalosporins resistance in Salmonella [149].

The most common resistance mechanisms were due to plasmid-mediated cephalosporinases/AmpC’s (plasmid-mediated ampC’s) and class A ESBL’s. Of these mechanisms the plasmid-mediated CMY-2 gene is the most widely disseminated β-lactamase in \textit{Salmonella} spp. [149]. \textit{S. Typhimurium} and \textit{S. Enteritidis} are the most common serotypes associated with extended-spectrum cephalosporins resistance in human infections [149].

Non-typhoidal salmonellae harbouring CTX-M enzymes have been reported in Europe, South America, and Asia [150]. CTX-M producing Salmonella have been detected in humans in the United Kingdom with an increasing number being detected in food animals in the UK and more generally in the European Union [151]. The most frequently isolated CTX-M genes in Salmonella from clinical sources are CTX-M-15 (CTX-M Group 1) and CTX-M-9 (CTX-M Group 9) with no reports to date of CTX-M Groups 8 or 25 like genes in Salmonella. CTX-M Group 2 like ESBLs (CTX-M-2, 4, 5) are less frequently
detected in *Salmonella* spp. Extended-spectrum cephalosporin resistance caused by CTX-M-2 in *S.* Virchow has been reported in Belgium from poultry and human samples, with the authors theorising that the strains causing illness in humans were introduced into the food chain by contaminated poultry products [152]. *bla*<sub>CTX-M-Group 2</sub>-like genes have also been detected in *S.* Typhimurium strains from Buenos Aires, Argentina [153], Russia and Belarus [154]. In addition, there have been isolated reports of other CTX-M genes occurring in non-typhoidal Salmonella from clinical samples [149]. Most recently, CTX-M-37 was detected in a *S.* Insangi from a paediatric patient in Durban, South Africa [155] and CTX-M-57 was detected in a *S.* Typhimurium from a patient in the UK with associated travel to Thailand [156]. ESBL-producing Salmonella from humans was first reported in Ireland in 2002. The serotypes identified producing ESBLs were *S.* Worthington (TEM-1 + SHV-12), *S.* Typhimurium (CTX-M-14), *S.* Typhimurium (CTX-M-15), *S.* Typhimurium (Unknown ESBL gene) and *S.* Concord (CTX-M-15 + TEM-1) [157].

OXA-1 is commonly reported within the variable region (VR) of class 1 integron cassettes in conjunction with the aminoglycoside resistance gene *aadA1*. This OXA-1-*aadA1* integron type has been reported in clinical isolates of *E. coli* [127] [158] *Shigella* spp.[123, 127, 128] and *Salmonella* spp. [118, 159-167] from different countries across the globe.

### 1.6.2 β-lactamases and ESBLs in Salmonella of animal origin

The literature indicates that food animals are increasingly reported as being associated with pathogen’s harbouring ESBL’s [168]. In the United States *S. enterica* isolates with reduced susceptibility to ceftriaxone have been detected in cattle [169]. In the UK the first report of an ESBL from livestock was reported in 2006 [151], with the ESBL responsible being *bla*<sub>CTX-M</sub> <sup>17/18</sup> from an *E. coli* isolated from calves. The problem has also been detected in Portugal with the observation of *bla*<sub>CTX-M</sub> <sup>9</sup> in *S.* Virchow from laying hens and *S.* Enteritidis in broilers, with *bla*<sub>SHV-12</sub> being identified in *S.* Rissen from pigs [170]. Multidrug-resistant *S.* Kentucky isolated from the carcasses of slaughtered pigs was reported in Ethiopia in 2006 [171].
It is important to highlight that cephalosporins are not licensed for use in poultry production in Ireland; however, amoxicillin is used for the control of clostridia and bacterial enteritis and therefore may generate a selective pressure for possession and retention of a β-lactamase. The occurrence and persistence of β-lactamase-producing Salmonella on farms may be related to antimicrobial prescribing [172-175].

1.6.3 The role of integrons and insertion sequences in dissemination of β-lactamase genes

Integrons were initially discovered in bacterial isolates in the clinical setting, however, it has since been established that approximately 10% of already sequenced bacterial genomes harbour these genetic elements [176]. It is therefore evident that integrons have been vital in microbial evolution and have enabled bacteria to adapt and survive to various environmental challenges [177]. Integrons may be located on plasmids or on transposons and therefore possess the capability for chromosomal insertion. Class 1 Integrons (C1I) are the most common integrons associated with antimicrobial resistance gene cassettes in the *Enterobacteriaceae*. C1I are particularly relevant in the clinical context as their gene cassettes commonly encode genes for resistance to commonly administered antimicrobial agents.

C1I are the most common class of integrons detected in Salmonella [178]. During the last decade’s multi-drug resistant (MDR) *S. enterica* of various serotypes have spread across the globe. This trend has been seen with *S. Typhimurium* DT104 with a MDR profile of resistance to Ampicillin, Chloramphenicol, Streptomycin, Sulphonamide and Tetracycline (ACSSuT) (encoded on Salmonella Genomic Island, SGI1) [179], with *S. Choleraesius* and with monophasic *S. Typhimuirum* carrying hybrid plasmids conferring resistance and virulence traits [178, 180-183]. More recently C1I’s have been recognised in many other serotypes of *S. enterica* including *S. Enteritidis*, *S. Brandenburg*, and *S. Virchow* [161].
Class 1 Integrons harbouring the gene cassette $\text{bla}_{\text{OXA-1}}$/aadA1 have recently emerged as important resistant determinants in $S$. Typhimurium in Spain, Italy and Portugal. The first report of this integron profile was from $S$. Typhimurium isolated from infants suffering acute gastroenteritis in Italy in the late 1990’s [184]. There have only been sporadic reports of this integron profile in $S$. Typhimurium isolated in various European countries since 1998 [42, 161, 167, 178]. However, a recent report by Pérez-Moreno and colleagues stated that this integron profile is becoming endemic in Spain and is rapidly moving pandemic SGI1-harbouring $S$. Typhimurium to second place [164, 185].

Insertion sequences (IS) form integral portions of bacterial chromosomes, where they have been shown to participate in chromosome rearrangements and in plasmid DNA integration/recombination events [186]. IS elements have been shown to regulate the expression of neighbouring genes [186, 187]. Insertion into the target DNA sequence occurs upon recognition of a sequence which is specific for the IS element in question [186]. There are a variety of insertion elements associated with antimicrobial resistance genes in Salmonella spp (IS elements belonging to the following families-IS6, IS10, IS26, IS605 and ISAs1). For the purpose of this review I will introduce those most frequently associated with antimicrobial resistance towards the $\beta$-lactam antimicrobials in Salmonella-IS$Ecp1$ and IS$CR$ elements.

$\text{ISEcp1}$ is a single copy insertion sequence which is responsible for mobilization of certain $\text{bla}$ genes, in particular this IS element has been identified upstream of several $\text{bla}_{\text{CTX-M}}$ genes [188]. More recently $\text{ISEcp1}$ has been identified in Salmonella strains harbouring the ESBL $\text{bla}_{\text{CTX-M-15}}$ in Kuwait [189] and the ESBL $\text{bla}_{\text{CTX-M-5}}$ in the USA [121]. Interestingly, Lartigue and colleagues illustrated that the $\text{bla}_{\text{CTX-M-2}}$ progenitor in Kluyvera ascorbata could be efficiently mobilized and transferred to a conjugative $E$. coli plasmid by the $\text{ISEcp1}$ element with enhanced mobilization observed in the presence of ceftazidime, cefotaxime, and piperacillin [187]. Insertion sequence common regions (IS$CR$’s), which are thought to have been derived from 1$S91$-like insertion elements, are becoming more frequently linked with the mobilization,
dissemination and expression of β-lactamase genes in various members of the Enterobacteriaceae. ISCRs were first discovered and reported in the early 1990’s as a DNA sequence of 2,154 bp that was found in two complex C1I’s called In6 and In7 [190]. ISCR elements have been implicated in the formation of the MDR region of the Salmonella Genomic Island (SGI1) and its variants. Therefore, ISCRs have significance in the mobilization of adjacent antimicrobial resistance genes and potentially pose a considerable threat to treatment of infections, particularly those caused by Gram-negative bacteria.

1.7 Antimicrobial susceptibility testing

1.7.1 Standardisation of antimicrobial susceptibility testing in clinical and veterinary microbiology

In Europe there are approximately seven antimicrobial susceptibility testing standards and the particular standard adhered to is dependent on the country, for example: in the United Kingdom- the British Society for Antimicrobial Chemotherapy (BSAC) working party on Antimicrobial susceptibility testing, in France there is the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM), in Germany the Deutsches Institut für Normung (DIN), in the Netherlands the Commissie Richtlijnen Gevoeligheidsbepalingen (CRG), in Norway the Norwegian Working Group on Antibiotics (NWGA), and in Sweden the Swedish Reference Group on Antibiotics (SRGA). The CLSI (formerly the NCCLS, National Committee for Clinical Laboratory Standards)) is accredited by the American National Standards Institute (ANSI) (www.clsi.org).

In the absence of a national system; or until recently, a European system, many laboratories in Europe have followed CLSI standards [191]. Therefore, there was a need for harmonisation of antimicrobial susceptibility standards in Europe with clear concise interpretative criteria available. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) was established to meet this need. In 2009 EUCAST published its first breakpoint table for interpretation of MIC’s and zone diameters [192], with the most recent version published in 2012 [193].
The clinical microbiology laboratory is important in detecting and reporting ESBL-production; however in some instances staff may not be fully up to date on the current methods for antimicrobial susceptibility testing. A US study reported that only 8% of clinical laboratories from rural hospital areas routinely screened for ESBL-producing organisms and in Italy 50% of clinical laboratories mis-identified ESBL-producing isolates [143].

1.7.2 ESBL detection in the clinical laboratory

In the CLSI standard M100-S21 the breakpoints for cefazolin, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone and aztreonam were updated [194] in accordance EUCAST and CLSI views that there was a need to reduce many of the cephalosporin breakpoints [195]. The EUCAST breakpoints for ampicillin, ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanate, piperacillin-tazobactam, cefotaxime, ceftibuten, imipenem and tobramycin have also been recently updated [193].

One of the major issues facing both the CLSI and EUCAST was whether or not to recommend reporting antimicrobial susceptibility test (AST) results prior to confirming ESBL production. Delay in administering antimicrobial therapy can have a significant impact on mortality rates and hospital stay costs [196] [143]. The CLSI in M100-S21 state that ‘when using the new interpretative criteria, routine ESBL testing is no longer necessary before reporting cephalosporin, penicillin or aztreonam results (i.e, it is no longer necessary to edit results for cephalosporins, aztreonam or penicillins from susceptible to resistant).’ EUCAST, Version 2.0, Note 1 states ‘The cephalosporin breakpoints for Enterobacteriaceae will detect all clinically important resistance mechanisms (including ESBL and plasmid-mediated AmpC). Some isolates that produce beta-lactamases are susceptible or intermediate to 3rd or 4th generation cephalosporins with these breakpoints should be reported as tested, i.e. the presence or absence of an ESBL does not in itself influence the categorisation of susceptibility’. Therefore, according to EUCAST and CLSI, when following the updated interpretative criteria, it is no longer necessary to confirm ESBL production prior to reporting AST results for the purpose of guiding
antimicrobial therapy. Leclercq and colleagues have recently published a document entitled ‘EUCAST expert rules in antimicrobial susceptibility testing’ to supplement the current EUCAST guidance [197].

Both CLSI M100-S21 and EUCAST Version 2.0 2012 recommend that ESBL detection and characterisation is recommended for epidemiological and infection control purposes [193, 194].

In the 1980s it was realised that the same standardisation seen in human medicine for AST was required for veterinary practice [4]. In 1998, the CLSI formed a subcommittee on Veterinary Antimicrobial Susceptibility testing (V-AST). The V-AST of the CLSI began with the task of developing standardised methods and interpretative criteria for AST testing of pathogens in veterinary medicine [198]. The first of these standards were published in 1999 [199]. This has been recently replaced by the CLSI/NCCLS M31-A3-Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals [200, 201]

### 1.7.2.1 Phenotypic testing for β-lactamase production

CLSI recommend using at least one extended-spectrum cephalosporin, or for improved sensitivity in detection of ESBL production, a panel of antimicrobials (cefotaxime, ceftazidime, cefpodoxime, aztreonam or ceftriaxone) for routine screening of ESBL production in clinical isolates of *E. coli*, *K. oxytoca* and *K. pneumoniae*. Ceftazidime and cefpodoxime exhibit the highest sensitivity in screening for ESBL production as the majority of ESBLs confer resistance to these antimicrobials [202].

The Health Protection Agency (UK) QSOP51 states that: ‘*The ideal indicator cephalosporin is one to which all ESBLs confer resistance, even when their production is scanty*’[203]. QSOP 51 also states that TEM and SHV ESBLs are generally phenotypically more easily detected when using ceftazidime, CTX-M ESBLs when using cefotaxime with all ESBLs exhibiting resistance to cefpodoxime. Inappropriate indicators are cefuroxime, cephalaxin and
cephemadine. QSOP 51 recommends to use either cefpodoxime or both cefotaxime and ceftazidime when screening for ESBL production. An alternative suggestion is that when testing community urine samples cephalexin and cephradine may be used, however, all positive results require follow up confirmatory ESBL testing.

1.7.2.2. ESBL confirmation based on clavulanic acid synergy
The CLSI confirmatory test (broth dilution/ disk diffusion) involves using an extended-spectrum cephalosporin (usually cefotaxime or ceftazidime) with and without clavulanic acid. QSOP 51 ESBL confirmatory testing recommends using any of three following confirmatory tests: double disk diffusion tests, combination disk test or Etest ESBL strips.

1.7.2.2.1 Combination disk diffusion and broth micro dilution
An increase of ≥5mm in the diameter of the zone of inhibition for the chosen extended-spectrum cephalosporin plus clavulanic acid compared to the diameter for the extended-spectrum cephalosporin alone is considered indicative of ESBL production when using the CLSI disk diffusion method. A decrease of ≥3 doubling dilutions (i.e. at least 8 fold) in an MIC of the chosen extended-spectrum cephalosporin plus clavulanic acid (4μg/ml) compared to extended-spectrum cephalosporin alone is indicative of ESBL production when using the CLSI broth micro dilution protocol. It is, however, difficult in some instances to detect the presence of ESBL in bacteria that simultaneously express an ampC gene. Because AmpC is not inhibited by clavulanic acid these enzymes may mask the ESBL and therefore the isolate does not show clavulanic acid enhancement. This is seen in E. cloacae, which produce an intrinsic AmpC β-lactamase [204].

Further complications that may arise in phenotypic ESBL confirmation is in relation to K. oxytoca. Approximately 10-20% of K. oxytoca possess a chromosomal β-lactamase called K1. Hyper-production of this enzyme results in resistance to cefpodoxime and cefotaxime but sensitivity to ceftazidime. Synergy with clavulanic acid is observed with cefepime and ceftotaxime (not ceftazidime) in these situations and therefore a false positive result may be obtained.
1.7.2.2.2 AB Biodisk Etest® ESBL Strips

These are plastic strips that contain an increasing gradient of an extended-spectrum cephalosporin on one end and extended-spectrum cephalosporin plus clavulanic acid on the other (AB Biodisk, Solna, Sweden). Greater than or equal to an 8-fold reduction in the MIC of the ESC tested when compared to the extended-spectrum cephalosporin in conjunction with clavulanic acid is indicative of ESBL production. The cefepime (PM), cefepime/ clavulanic acid (PML) Etest® synergy test (PM/PML) is generally reserved for screening for ESBL production in isolates that co-produce AmpC β-lactamases. This is because cefepime is relatively resistant to hydrolysis by AmpC β-lactamases. Stürenburg and colleagues have reported that the PM/PML synergy test is actually more sensitive than the cefotaxime-clavulanic acid (CT/CTL) and the ceftazidime-clavulanic acid (TZ/TZL) Etest® synergy tests for the detection of ESBL production in a collection of ESBL-producing Enterobacteriaceae [205]. BSAC recommends using the PM/PML synergy test in confirmation of ESBL production in Enterobacter spp., Morganella spp., Providencia spp., Citrobacter spp. and Serratia spp. (i.e. inducible chromosomal AmpC). The CA-SFM recommends using PM/PML in all Enterobacteriaceae that possess a naturally occurring AmpC on their chromosome [97]. Oxoid currently offer a similar product to the AB Biodisk Etest®. It is called the M.I.C. Evaluator (M.I.C.E) strip. However, the only synergy test offered is the amoxicillin/ clavulanic acid M.I.C.E strip.

1.7.2.2.3 Automated ESBL confirmation based on clavulanic acid synergy

The Vitek 2 (bioMérieux, Hazelwood, Mo.) ESBL test allows simultaneous assessment of the inhibitory effects of cefotaxime, ceftazidime and cefepime extended-spectrum cephalosporin alone and in conjunction with clavulanic acid. The BD Phoenix ESBL test is similar to the Vitek 2 ESBL system. The BD Phoenix (Becton Dickinson) is an automated AST platform that is based on simultaneous assessment of the inhibitory effects of cefotaxime, ceftazidime, cefpodoxime and aztreonam (alone or in combination with clavulanic acid).
cefotetan ± boronic acid (AmpC test), and ceftazidime ± clavulanic acid ± boronic acid (masked AmpC detection).

Phenotypic tests to detect class C β-lactamases involve cloxacillin synergy. A major issue is the lack of widely accepted phenotypic tests to detect Class D OXA β-lactamase producing Enterobacteriaceae in the laboratory. OXA-13 and OXA-19 are inhibited by imipenem (but not by clavulanic acid) and the placement of an imipenem disk close to a cefsulodin disk enables the detection of these enzymes [104].

1.7.3 Molecular techniques for β-lactamase detection

Molecular methods are required in order to unequivocally confirm the presence of a particular ESBL-encoding gene in the test organism.

The use of simplex or multiplex PCR’s can be applied to detect specific ESBL genes. Woodford recently published a chapter in Methods in Molecular Microbiology outlining protocols for the detection of numerous β-lactamase genes by multiplex PCR [206]. One of these multiplex PCR target’s the CTX-M ESBL’s (groups 1, 2, 8, 9 & 25), another PCR targets the commonly encountered metallo-β-lactamase genes (IMP, VIM, GIM, SPM & SIM) and there is also a protocol for the detection of the commonly found OXA carbapenemases (OXA-51-like, OXA-23-like, OXA-40-like and OXA-58-like). Dallenne and colleagues have also recently published PCR protocols for the detection of routinely encountered class A ESBLs, plasmid-mediated AmpC’s, OXA-1-like broad-spectrum β-lactamases, and class A, C & D carbapenemases [207]. Multiplex one of Dallenne’s protocol detects TEM, SHV and OXA Group III like β-lactamases, multiplex II detects CTX-M groups 1, 2, 8, 9 & 25, multiplex 3 detects plasmid-mediated AmpC’s (ACC, MOX, FOX, DHA, CIT & EBC), multiplex 4 detects VEB, PER & GES variants, multiplex 5 detects GES variants & OXA-48, and multiplex 6 detects IMP, VIM and KPC variants.

Grimm and colleagues in 2006 validated a DNA microarray to identify 96% of the TEM variants based on single nucleotide polymorphisms (SNP) [208]. The
total assay time, including PCR, hybridization, and image analysis is less than 4 hours. Stuart and colleagues in 2009 presented their study at the 19th European Congress of Clinical Microbiology and Infectious Disease in Helsinki, Finland on a novel DNA microarray for the rapid detection of TEM, SHV and CTX-M ESBLs in Enterobacteriaceae [209]. They concluded that the microarray was an attractive and accurate option for rapid detection of TEM, SHV and CTX-M producing Enterobacteriaceae. Leinberger and colleagues in 2009 (the same group that developed the TEM –variant microarray, [208]) also reported on constructing and validating a DNA microarray for rapid detection and genotyping of TEM, SHV and CTX-M genes in Gram-negative bacteria. This microarray chip was an advanced chip containing an updated version of the TEM-microarray with additional regions for genotyping SHV and CTX-M genes [210]. This chip is able to detect of isolates harbouring multiple ESBL genes within 5 hours. The sensitivity of the microarray was reported to be 93%.

Recently, Check-points BV (Wageningen, The Netherlands) have launched two commercially available microarray platforms for detection of the most commonly encountered β-lactamases (http://www.check-points.com/ ). One is termed the Check-MDR-CT101 array (for the detection of NDM-1, KPC, CTX-M-groups, TEM-variants, SHV-variants, CMY, DHA, FOX, MOX, ACC, MIR & ACT) array and the other the Check-MDR-CT102 array (for the detection of NDM-1, VIM, IMP, KPC, OXA-48, TEM-variants, SHV-variants, CTX-M-groups). Naas et al., Cohen- Stuart et al., Endimiani et al. and Woodford et al. have each independently evaluated each of these microarray platforms [211-215]. These arrays were shown to be able to detect TEM/ SHV β-lactamase and TEM/SHV-ESBL variants simultaneously present within the same isolate. In addition the microarray platform allowed for identifying β-lactamases/ESBLs in isolates that co-produced K1 (K. oxytoca) or chromosomal AmpC (e.g. Enterobacter spp.). The entire procedure can be carried out in less than 8 hours for 50 isolates with a sensitivity/ specificity rate for SHV variants of 98.8%/ 100%, for TEM variants of 100%/ 96.4% and for CTX-M and KPC variants of 100%/100% [212]. The check point array possesses regions for 95% of the TEM variants [66] and regions for 75% of the SHV variants [66] [211].
Leinberger array encompassed regions for 99% of TEM variants and 94% of SHV variants. However, as of yet, the Leinberger array is not commercially available [210].

1.8 Shotgun cloning

A major objective of DNA cloning is to obtain discrete, manageable (small) regions of a genetic element. The following information is based upon information in Sambrook and Russell, Molecular Cloning - A Laboratory Manual [216].

Restriction enzymes are endonucleases produced by bacteria that typically recognise specific 4-8 base pair sequences called restriction sites. The restriction enzyme digests the DNA at this point. Restriction sites commonly are short palindromic sequences. Restriction enzymes can make staggered cuts in a DNA sequence at the recognition site which results in a single stranded ‘tail’ at both ends, e.g. *EcoRI*. The tails on the fragments generated at a given restriction site are complementary to all other fragments generated by the same restriction enzyme. These single stranded regions (‘sticky’ ends) can base pair with those on other DNA fragments generated by the same restriction enzyme. Some restriction enzymes cleave DNA to generate fragment ends termed ‘blunt ends’ e.g. *SmaI*.

Another enzyme involved in DNA cloning is DNA ligase. DNA fragments with either ‘sticky’ or ‘blunt’ ends can be inserted into the vector of choice with the aid of DNA ligases. During normal DNA replication, DNA ligase catalyses the end to end joining of (ligation) of short fragments of DNA called Okazaki fragments. For the purpose of cloning technology, purified DNA ligase is utilised to covalently join the ends of a restriction fragment and vector DNA that possess complementary ends. The vector and restriction fragment are covalently

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8 A palindromic sequence is where the restriction site specific sequence is the same on each DNA strand when read in the 5’-3’ direction

9 Nucleotides are added by a DNA polymerase to each growing daughter strand in the 5’-3’ direction during DNA replication. The ‘lead’ strand is synthesised from a singled RNA primer. The ‘lagging’ strand is synthesised discontinuously from multiple RNA primers that are formed periodically as each new region of the template parental DNA duplex unwinds. Elongation of these RNA primers produces short fragments termed ‘Okazaki’ fragments named after their discoverer Reiji Okazaki.
ligated together through 3’-5’ phospho-diester bonds of DNA. In addition to ligating complementary ‘sticky’ ends DNA ligase from bacteriophage T4 can ligate any two ‘blunt’ ends.

Shotgun cloning is a cloning strategy that involves digestion of test DNA and the ligation of the resulting randomly sized fragments into a vector. The outcome is a large library of recombinant plasmids each containing a different fragment. Vector transfer into a host cell can be achieved in the laboratory by electroporation. Electroporation involves exposing host cells to pulsed electric fields in the presence of cloned DNA. There are several methods devised to distinguish bacteria transformed by recombinant plasmids from those carrying the wild type plasmid. The most durable and widely used of these methods uses a non-destructive histo-chemical technique to detect β-galactosidase (encoded by lacZ) activity in transformed bacteria. This is called α-complementation. The α-complementation procedure is used to distinguish colonies of cells that carry recombinant plasmids from those that do not.

α-Complementation occurs when two inactive fragments of E. coli β-galactosidase associate to form a functional enzyme. Deletion of the 5’ region of the lacZ gene generates a carboxy terminal fragment of the β-galactosidase enzyme called the ω or α-acceptor fragment. An amino terminal fragment (α-donor fragment) is generated by deletion or mutation of the lacZ gene. Neither the ω/ α-acceptor fragment nor the α-donor fragment are enzymatically active, however, both can associate to form an active β-galactosidase enzyme in vitro. Many plasmid vectors carry a short segment of E. coli DNA containing the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene. Embedded in this coding region is a polycloning site that maintains the reading frame and results in incorporation of a small number of amino acids into the amino terminal fragment of β-galactosidase. Cloning vectors with this coding region are used in host cells that express the carboxy terminal fragment of the β-galactosidase enzyme. Although neither the host cell-encoded nor the plasmid-encoded fragments of the β-galactosidase are themselves active, they can associate to form an enzymatically active protein. In
most bacterial strains used for α-complementation, the ω/ α-acceptor fragment is encoded by the deletion mutant lacZΔM15, which lacks codons 11-41 of the β-galactosidase gene. The lac+ bacteria that result from α-complementation are easily recognised as they form blue colonies in the presence of the chromogenic substance X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside). The β-galactosidase enzyme converts X-gal into an insoluble dense blue compound. Insertion of a fragment of DNA into the poly cloning site of the plasmid vector results in production of an amino terminal fragment that is no longer capable of α-complementation. Bacteria harbouring these recombinant plasmids will appear as white colonies on agar containing X-gal. The structure of the recombinant plasmid can be assessed by restriction analysis and sequencing of the inserted fragments.

IPTG (isopropyl- β-D-thiogalactoside) is a non-fermentable analogue of lactose that inactivates the lacZ repressor and therefore induces transcription of the lac operon (encodes genes responsible for the expression of the lacZ gene). Structural analogues may induce or repress expression even though they themselves are not substrates for the specific enzyme. IPTG is used in molecular cloning to induce synthesis of both fragments of the β-galactosidase enzyme. One of the most common plasmid vectors used in molecular cloning are those derived and genetically engineered from the pUC vectors. The site of insertion of foreign DNA into pUC vectors occurs at the amino terminus of the α-donor fragment of the β-galactosidase gene lacZ, the promoter associated with pUC vectors is the lac promoter and insertion of foreign DNA into the multiple cloning site (MCS) abolishes α-complementation.
1.9 Aim of Thesis

The primary aim of my thesis was to characterize the genetic basis for a novel ESBL phenotype observed in a collection of *Salmonella enterica* subspecies *enterica* serotype Typhimurium from Kenya, Ireland and Malawi. A working term ‘cefepimase’ was used to describe this phenotype. ‘Cefepimase’ producing *Salmonella* Typhimurium were defined as having resistance to cefepime, reduced susceptibility to cefotaxime and susceptibility to ceftazidime. Cefepimase producers did not confirm as ESBL producers using the CLSI ESBL confirmatory criteria (ceftazidime ± clavulanic acid, cefotaxime± clavulanic acid). However, ESBL production was suspected due to synergy observed between cefepime and clavulanic acid.

A secondary aim of my research was to analyse the genetic basis of β-lactamase resistance and epidemiological relatedness of a collection of geographically distinct *Salmonella enterica* subspecies *enterica* serotype Kentucky isolated from poultry production sites in the Republic of Ireland.
Characterization of a novel extended-spectrum β-lactamase phenotype from OXA-1 expression in Salmonella enterica serovar Typhimurium strains from Africa and Ireland.
2.1 Abstract

OXA-1 is reported as not being inhibited by clavulanic acid. In this study we report an ESBL phenotype observed in *Salmonella enterica* resulting from the high level expression of *bla*<sub>OXA-1</sub>. This phenotype was observed in *S. Typhimurium* isolates from Kenya, Malawi and Ireland. All African isolates were identified as MLST sequence type ST313, with the Irish isolate identified as ST19.

2.2 Introduction:

Extended-spectrum β-lactamases (ESBLs) confer resistance to the penicillins and oxyimino-cephalosporins and are inhibited by the β-lactamase inhibitors (e.g. clavulanic acid). ESBLs have been reported worldwide with increasing frequency and diversity in members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa*. ESBL production in *Salmonella* spp. was first reported in 1988 [148] and is increasing in prevalence worldwide [217]. For further information on Non-typhoid salmonellae refer to Chapter 1 Section 1.1.1.

For information on β-lactamase in Salmonella refer to Chapter 1 section 1.6. In addition, as discussed in Chapter 1 section 1.7, there are a number of phenotypic methods for detection of ESBL production. Although detection of the mechanism may be less important for therapeutic purpose now than formerly, detection remains useful for infection control and public health purposes.

We have identified a collection of *S. Typhimurium* that exhibit preferential resistance to cefepime (compared with cefotaxime or ceftazidime) and with marked reduction of the cefepime MIC in the presence of clavulanic acid. In this paper we describe the genetic basis for the emergence of this unusual resistance phenotype.
2.3 Materials and methods:
A detailed list of the materials, equipment and methods used for this work are documented under ‘Chapter 2 Materials, methods and equipment’ on attached CD disk.

2.3.1 Bacterial strains:
A collection of 19 isolates of *Salmonella enterica* serovar Typhimurium showed a phenotype of high cefepime MIC (8-32 µg/ml) relative to cefotaxime (0.25-2.0 µg/ml) and ceftazidime MIC (0.5 µg/ml). The cefepime MIC was markedly reduced (0.064-0.25 µg/ml) in the presence of clavulanic acid. The collection comprised 17 isolates collected from blood cultures of patients in Nairobi, Kenya [218], one isolate from Malawi (PB-1052) and one (NSRL 227) isolated from the faeces of a patient in Ireland, with associated travel to Andorra [157]. It is important to note that it is not known if the samples from Sub Saharan Africa were obtained from AIDS patients. Refer to Table 2.1 and Table 2.2 for details on the collection of bacterial isolates. Table 2.1 details the total number of *S.* Typhimurium available for analysis, the numbers of β-lactamase producers possessing a normal phenotype and the number of *S.* Typhimurium identified to harbour a novel ESBL phenotype. Table 2.2 presents detailed information on the *S.* Typhimurium possessing the novel ESBL phenotype.
Table 2.1 The total number of the total number of *S. Typhimurium* available for analysis, the numbers of β-lactamase producers possessing a normal phenotype and the number of *S. Typhimurium* identified to harbour a novel ESBL phenotype.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Total numbers of <em>S. Typhimurium</em> isolated</th>
<th>Total number of β-lactamase producers identified</th>
<th>Total Number of ‘cefpimase’ producers identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ireland (2000-2011)</td>
<td>10,500</td>
<td>57</td>
<td>1</td>
</tr>
<tr>
<td>Kenya (1994-2003)</td>
<td>300</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Malawi (2002)</td>
<td>50</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.2 Bacterial test strains exhibiting the ‘cefepimase’ phenotype

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Isolate #</th>
<th>Year of isolation</th>
<th>Country</th>
<th>Source</th>
<th>Sample</th>
<th>Phage type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typhimurium</td>
<td>PB-1052</td>
<td>2002</td>
<td>Malawi</td>
<td>Human</td>
<td>Blood</td>
<td>DT56 Var</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>100</td>
<td>1994-2003*</td>
<td>Kenya</td>
<td>Human</td>
<td>Blood</td>
<td>DT193</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>165</td>
<td>1994-2003*</td>
<td>Kenya</td>
<td>Human</td>
<td>Blood</td>
<td>DT56var</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>246</td>
<td>1994-2003*</td>
<td>Kenya</td>
<td>Human</td>
<td>Blood</td>
<td>Untypable</td>
</tr>
<tr>
<td>Typhimurium</td>
<td>NSRL-227**</td>
<td>2005</td>
<td>Ireland</td>
<td>Human</td>
<td>Faeces</td>
<td>Untypable</td>
</tr>
</tbody>
</table>

* Isolates were submitted to the NSRL for typing, data available upon submission to the NSRL is that the *Salmonella* were collected during the period of 1994-2003. ** Associated travel to Andorra.
2.3.2 Antimicrobial susceptibility testing:
ESBL production was confirmed by the combination disk method of CLSI using cefpodoxime (30µg), and cefpodoxime plus clavulanic acid (10µg/1µg) and by the ESBL Etest method using ceftazidime/ceftazidime plus clavulanic acid (TZ/TZL); cefotaxime/cefotaxime plus clavulanic acid (CT/CTL); and cefepime/cefepime plus clavulanic acid (PM/PML) Etest strips in accordance with the manufacturers’ instructions (AB Biodisk, Solna, Sweden). All ‘cefepimase’ producers were tested for susceptibility to the following antimicrobial agents in accordance with CLSI disk diffusion methods: ampicillin (10µg), chloramphenicol (30µg), streptomycin (10µg), sulphonamides (300µg), tetracycline (30µg), trimethoprim (5µg), nalidixic acid (30µg), ciprofloxacin (5µg), gentamicin (10µg), kanamycin (30µg), minocycline (30µg), aztreonam (30µg), meropenem (10µg), cefpirome (30µg), ceftazidime (30µg), cefoxitin (30µg), cefuroxime (30µg), and rifampicin (30µg) [219]. All antimicrobial disks were received from Oxoid, Basingstoke, U.K. The minimum inhibitory concentration (MIC) of cefepime for all “cefepimase” producers was determined by agar dilution and broth micro dilution in accordance with the CLSI ‘Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved Standard-Seventh Edition’ [220].

2.3.3 DNA extraction of bacterial isolates:
DNA extraction was carried out on all bacterial isolates using the QIAamp DNA Mini Kit (Qiagen Inc. Valencia Calif) in accordance with the manufacturer’s instructions. DNA extracts were stored prior to molecular work at -20°C.

The DNA extraction protocol is documented under ‘Protocols-Protocol # 1’ on attached CD disk.
2.3.4 PCR amplification

PCR amplification conditions used are documented in ‘Chapter 2-PCR amplification conditions’ on attached CD disk.

All confirmed ESBL producers were screened for bla<sub>TEM</sub>, bla<sub>SHV</sub>, and bla<sub>CTX-M</sub> by PCR using specific primers and protocols reported previously (Table 2.3). All ‘cefepimase’ producers were additionally screened for the presence of Class I and Class II integrons and additional β-lactamase encoding genes by PCR using specific primers and protocols as previously described (Table 2.3). We investigated by PCR if there was a genetic link between bla<sub>OXA-1</sub> and a variety of insertion elements which have been reported to be associated with β-lactamase genes (IS<sub>CR1</sub>, IS<sub>1</sub>, IS<sub>26</sub>, IS<sub>Ecp1</sub>). Presence of the Salmonella genomic Island (SGI) was assessed using previously described protocols [221, 222]. Absence of inhibitors of PCR was confirmed in all DNA preparations by amplification of the 16S rRNA and 23S rRNA spacer region [223]. Strains encoding bla<sub>CTX-M-9</sub>, M-15, M-25, M-2, bla<sub>TEM-5</sub>, bla<sub>TEM-8</sub>, bla<sub>SHV-3</sub>, bla<sub>SHV-4</sub>, and bla<sub>OXA-1</sub> were used as positive controls for relevant amplification experiments and were kindly provided to us by Dr. Neil Woodford, Health Protection Agency, UK.
### Table 2.3 Primers used in PCR analyses of ‘cefepimase’ producing S. Typhimurium isolates

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA Space F: AGICGTAAACAGGATACCOCG</td>
<td>[223]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CTCAGCTTCGCTAAGACTCCACCC</td>
<td></td>
</tr>
<tr>
<td>blaTEM</td>
<td>F: ATGACCTTCAAGATTTTGGG</td>
<td>[224]</td>
</tr>
<tr>
<td></td>
<td>R: TTMCAATGCTTAACAGTACGAG</td>
<td></td>
</tr>
<tr>
<td>blaSFO</td>
<td>F: ATGGGAATATACGCGCTTCG</td>
<td>[224]</td>
</tr>
<tr>
<td></td>
<td>R: GTTACGGTCCTGACGTCGG</td>
<td></td>
</tr>
<tr>
<td>blaCGA</td>
<td>F: AAA AAT CAC TAC GCA GCC</td>
<td>[225]</td>
</tr>
<tr>
<td></td>
<td>R: ACC TAT TCT ATOGC AGC TT</td>
<td></td>
</tr>
<tr>
<td>blaRTG</td>
<td>F: CCA GCC TAA GAT TAT TTT TGC</td>
<td>[225]</td>
</tr>
<tr>
<td></td>
<td>R: CCA GCC TAA GAT TAT TTT TGC</td>
<td></td>
</tr>
<tr>
<td>Class II integron F: GCC AAA GGC AGA TAT AGC</td>
<td>[226]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AAG CAG ACT TGA CCT GA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: TCT TAC GAC TCC AGC TGC</td>
<td>[226]</td>
</tr>
<tr>
<td></td>
<td>R: CCA ATG ACC CCC TCA CTT TCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: GCC AAA GGC AGA TAT AGC</td>
<td>[226]</td>
</tr>
<tr>
<td></td>
<td>R: AAG CAG ACT TGA CCT GA</td>
<td></td>
</tr>
<tr>
<td>Class II integron F: GGGGATCCCGGACGGCATG</td>
<td>[226]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCG TCC GAG TTG ACT GCC GG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: GCC AAA GGC AGA TAT AGC</td>
<td>[226]</td>
</tr>
<tr>
<td></td>
<td>R: AAG CAG ACT TGA CCT GA</td>
<td></td>
</tr>
</tbody>
</table>

Note: Primers were examined using one primer specific to the IS element of interest and one primer specific to the 16S rRNA region. Typhimurium isolates were examined using one primer specific to the IS element of interest and one primer specific to the 16S rRNA region.
### Table 2.3 continued. Primers used in PCR analyses of ‘cefepimase’ producing *S. Typhimurium* isolates

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| \(\text{bla}_{BES-1}\)    | F: GAAAAACTCGAAGCCCCCTGCT  
R: CCAAGCTTGGCCCCTACCTG | [239]     |
| \(\text{bla}_{OXA-18}\)   | F: ACTGGCGGAATCATACACAAAC  
R: ATCCTTCGAAAAACCCGCA | [240]     |
| \(\text{bla}_{OXA-5}\)    | F: AGCCCTACGATCAAAATGATA  
R: AAAGCATCGACAAGATTGAAG | [235]     |
| \(\text{bla}_{OXA-20}\)   | F: AGAGCGGTGACTACTGGATAA  
R: AAAGCATCGACAAGATTGAAG | [235]     |
| \(\text{bla}_{OXA-45}\)   | F: CGCGGTAAACACACTGTCATA  
R: AGAACCTTTTCGCGAGATCC | [241]     |
| \(\text{bla}_{OXA-35}\)   | F: TGCGTGTCTTTCAAGTACGG  
R: CCCTCACTTGCCATGATTTT | [242]     |
| \(\text{bla}_{OXA-9}\)    | F: GAAACACCAATACTGCTCAT  
R: AGAACCTTTTCGCGAGATCC | [243]     |
| \(\text{bla}_{OXA-28}\)   | F: TGCGTGTCTTTCAAGTCGG  
R: CCCTCACTTGCCATGATTTT | [244]     |
| \(\text{bla}_{OXA-51-}\)  | F: CTAATTGCCTTTGACAGCTCA  
R: TGGATCTTACAAAAACCCGCA | [245]     |
| \(\text{bla}_{OXA-23-}\)  | F: GATCGATTGAACTGCAAA  
R: ATGATCTTACAAAAACCCGCA | [245]     |
| \(\text{bla}_{OXA-24-}\)  | F: GGTGATTTGGGCACTAGGAA  
R: AGTCCTTACAAAAACCCGCA | [245]     |
| \(\text{bla}_{OXA-58-}\)  | F: AAGTATTTGCTCGGTTTACAGT  
R: CAACTGCTGCTCTATCCAC | [246]     |
| \(\text{bla}_{PER-1}\)    | F: AATTTGCGTCTCTGTCGATCG  
R: ATGAGGAAGTTTTCACATCC | [226]     |
| \(\text{bla}_{PER-2}\)    | F: TGTGTTCAGCACTCCTTTATGAC  
R: GGCGCCTGTGTGCTCGAATACAT | [246]     |
| \(\text{bla}_{LCR-1}\)    | F: CCTTTGTCTCTTTTTTGTACAGT  
R: CGTCTCTTGATGCTCAGCTA | [235]     |
| Primer Walking C1H       | F: CGGATGGTTTGGTTCTAAGGTGGTTA  
R: AGGCCAATGCTTCCTAAGGTGGTTA | This Study |
| Control primer #1 (100 ng/μl) | CCATGATTACGCCAAGCGCGCAATTAACCCTCAC | Stratagene |
| Control primer #2 (100 ng/μl) | GTGAGGGTTAATTGCGCTTTGGCTGTTGCGTAATACATG | Stratagene |
| Del1214s                  | TTTTCACTGCGTTGGTACGCGTGTTTTGCTGACGTGCTG | This Study |
| Del1216as                 | GAACGATAGACTGTTGCAAAGAACACATCAAACCCGGAATCA | This Study |
2.3.5 Characterization of strains

Phage-typing, plasmid profile analysis, pulsed field gel electrophoresis (PFGE) and variable number tandem repeat (VNTR) analyses were performed on all ‘cefepimase’ producing *S.* Typhimurium. Phage typing was performed in accordance with the methods of the Laboratory of Enteric Pathogens, Health Protection Agency, Colindale, London, United Kingdom [247]. Plasmid number and size was determined by an alkaline lysis method as previously described [216]. PFGE was performed using the CHEF Mapper XA (Bio-Rad, California) system following the standardized protocol of PulseNet with *XbaI* and *BlnI* (Roche, Basel, Switzerland) [38]. MLST analysis was performed as reported previously (Table 2.4) [50]. MLVA analysis was performed in accordance with previously described protocols (Table 2.5) [41].

Detailed protocols relevant to this section are documented under ‘Protocols-Protocol # 4, 5, 6’ on attached CD disk.
### Table 2.4 MLST Primer Sequences and expected amplicons sizes

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’-3’</th>
<th>Amplicon Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>thrA:F</td>
<td>GTCACGGTGATCGATCCCGGT</td>
<td>852bp</td>
</tr>
<tr>
<td>thrA:R</td>
<td>CACGATATTGATATTAGCCCG</td>
<td></td>
</tr>
<tr>
<td>thrA:R1</td>
<td>GTGCCGATACCGTGCGCGAC</td>
<td></td>
</tr>
<tr>
<td>thrA:sF</td>
<td>ATCCCGCCCGATCACATGAT</td>
<td></td>
</tr>
<tr>
<td>thrA:sR</td>
<td>CTCCACAGGCCCTCTTTCAG</td>
<td></td>
</tr>
<tr>
<td>purE:F</td>
<td>ATGTCTTCCCGCAATAATCC</td>
<td>510bp</td>
</tr>
<tr>
<td>purE:R</td>
<td>ATGTCTTCCCGCAATAATCC</td>
<td></td>
</tr>
<tr>
<td>purE:R1</td>
<td>TCATAGCCTCCCCCGCGGATC</td>
<td></td>
</tr>
<tr>
<td>purE:sF</td>
<td>CGAGAACGCAAACCTTGCTTC</td>
<td></td>
</tr>
<tr>
<td>purE:sR</td>
<td>CGCGGATCGAGGATTTCCAG</td>
<td></td>
</tr>
<tr>
<td>purE:sR1</td>
<td>GAACGCAAACCTTGCTCAT</td>
<td></td>
</tr>
<tr>
<td>sucA:F</td>
<td>AGCCACGGAGAGAGACGCTG</td>
<td>643bp</td>
</tr>
<tr>
<td>sucA:R</td>
<td>GGTGTGGATAACCGATACGTAC</td>
<td></td>
</tr>
<tr>
<td>hisD:F</td>
<td>GAAACGTTCCATCCGGCGAGAC</td>
<td>894bp</td>
</tr>
<tr>
<td>hisD:R</td>
<td>CTGAACGCTCCTCGTTTCTG</td>
<td></td>
</tr>
<tr>
<td>hisD:Sf</td>
<td>GTGGGTGTATATTCCGGCG</td>
<td></td>
</tr>
<tr>
<td>hisD:sR</td>
<td>GTGAAATCGCATTCCCAAAATC</td>
<td></td>
</tr>
<tr>
<td>aroC:F</td>
<td>CCTGACCACCTCCTGGCATATA</td>
<td>820bp</td>
</tr>
<tr>
<td>aroC:R</td>
<td>CCACACCGGATCGTGCGGCG</td>
<td></td>
</tr>
<tr>
<td>aroC:sF</td>
<td>GGCACCATGGATGGCCTGCT</td>
<td></td>
</tr>
<tr>
<td>aroC:sR</td>
<td>CATATGCGCCACAAATGTGTTG</td>
<td></td>
</tr>
<tr>
<td>hemD:F</td>
<td>ATGAGTATTCTGATCAACCCG</td>
<td>666bp</td>
</tr>
<tr>
<td>hemD:R</td>
<td>GAGGCTTTGAGTGACCGCTGCG</td>
<td></td>
</tr>
<tr>
<td>hemD:Sf</td>
<td>ATCCGATGCCCGCGCCCTC</td>
<td></td>
</tr>
<tr>
<td>hemD:sR</td>
<td>GACCAATAGCGACAGCAGTAG</td>
<td></td>
</tr>
<tr>
<td>dnaN:F</td>
<td>ATGAAATTTACCGGTGAACGTTG</td>
<td>833bp</td>
</tr>
<tr>
<td>dnaN:R</td>
<td>ATTTTCTCACCGAGGATGGC</td>
<td></td>
</tr>
<tr>
<td>dnaN:R1</td>
<td>CGCGGAAATTTTCATCGAG</td>
<td></td>
</tr>
<tr>
<td>dnaN:sF</td>
<td>CGATCTCGTGAACCTGCT</td>
<td></td>
</tr>
<tr>
<td>dnaN:sR</td>
<td>CCATCCACAGCTTTGAGGT</td>
<td></td>
</tr>
</tbody>
</table>

**Note 1:** For each PCR reaction 2 primers were used, forward and reverse. In a case where there was no amplicons one of the alternative primers were used i.e if using thrA:F and thrA:R and there were no amplicons, the alternative thrA:R1 was used.  

**Note 2:** For sequencing, each amplified gene was added to 2 wells of a 96 well plate. One well is to add the sequencing forward primer and the other well is to add the sequencing reverse primer.  

**Note 3:** For sucA the PCR primers are also used for sequencing.  

**Note 4:** For dnaN, the primer dnaN:R1 (if used in the PCR reaction, may not need to be if dnaN:F and dnaN:R work) is also used as the sequencing reverse primer.
### Table 2.5  MLVA Primer Sequences

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>STTR9-F</td>
<td>AGA GGC GCT GCG ATT GAC GAT</td>
</tr>
<tr>
<td>STTR9-R</td>
<td>CAT TTT CCA CAG CGG CAG TTT TTC</td>
</tr>
<tr>
<td>STTR5-seqF</td>
<td>TTA TTA TTC TGA GCA CCG C</td>
</tr>
<tr>
<td>STTR5-seqR</td>
<td>TGA TAC GCT TTT GAC GTT GC</td>
</tr>
<tr>
<td>STTR6-F</td>
<td>TCG GGC ATG CGT TGA AAA</td>
</tr>
<tr>
<td>STTR6-R</td>
<td>CTG GTG GGG AGA ATG ACT GG</td>
</tr>
<tr>
<td>STTR10-F</td>
<td>CGG GCG CGG CTG GAG TAT TTG</td>
</tr>
<tr>
<td>STTR10-R</td>
<td>GAA GGG GCC GGG CAG AGA CAG C</td>
</tr>
<tr>
<td>STTR3-seqF</td>
<td>GAA AAA CGC GCA AAA CTC TC</td>
</tr>
<tr>
<td>STTR3-seqR</td>
<td>GCC ACT GGT TGT CCT GTT CT</td>
</tr>
</tbody>
</table>
2.3.6 Transfer of cefepime resistance

Conjugal transfer of cefepime resistance was attempted by liquid, solid and filter mating assays as previously described [125, 248, 249]. A nalidixic acid resistant plasmid free *Salmonella* Dublin was used as recipient and ‘cefepimase’ producers representative of individual plasmid profiles were used as donors. Mating mixtures were plated onto Luria Bertani (LB) agar containing nalidixic acid (50μg/ml) or sodium azide (100μg/ml) supplemented with cefepime (4 μg/ml) after 4, 7, 20 and 48 hours incubation at 25°C, 30°C and 37°C.

2.3.7 BamHI Restriction fragment length polymorphism (RFLP)

These extracts were prepared using the QIAprep® Plasmid Mini kit. A detailed protocol is documented under ‘Protocols-Protocol # 7’ on attached CD disk.

2.3.8 Southern blotting and hybridization

Plasmid extractions were run on a 0.7% TAE gel and visualised with ethidium bromide staining. Size was estimated using a standard curve constructed from plasmids from strains, NCTC 50012 (72mDA), NCTC 50001(62mDA), NCTC 50083(126mDA) and NCTC 50005(26mDA). The pEK499 plasmid harbouring *E. coli* UK Strain A (*bla*OXA-1 positive) was used as a positive control in analysis (Karisk et al 2006). Plasmid DNA was transferred to a positively charged nylon membrane (Roche Diagnostics) by the Southern blotting technique [216]. DNA was then UV cross-linked (Chef Mapper). A 908-bp PCR generated Digoxigenin labelled probe (primers OXA Gp 1-Like F and OXA Gp-1 like R *(Table 2.3)*) was used to probe plasmids for OXA-group like β-lactamases.

A detailed southern blot and DIG-labelled hybridization protocol is documented under ‘Protocols-Protocol # 8’ on attached CD disk.
2.3.9 PCR Based Replicon Typing (PBRT)

PCR amplification conditions used are documented in ‘Chapter 2-PCR amplification conditions’ on attached CD disk.

PBRT was carried out using primers and protocols of Caratolli et al. [36], refer to Table 2.6. In order to generate PBRT positive controls, TA cloned replicons for each of the 18 most common plasmid Incompatibility (Inc) groups identified in Enterobacteriaceae were electroporated into Top10 electrocompetent cells and grown on 50µg/ml ampicillin agar. A purified 39kb plasmid (pFEP39), common to all isolates with the “cefepime” phenotype was prepared by extraction from an agarose gel using the QIAprep® Plasmid Mini kit. This plasmid was included in all PBRT assays.

The extracts were prepared as per-Protocol # 7’ on attached CD disk.
Table 2.6 PCR based replicon typing (PBRT) primer details

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’-3’</th>
<th>Target</th>
<th>Amplicon Size</th>
<th>PCR #</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI1 FW</td>
<td>ggagcgatgtgattaacttctgac</td>
<td>parA-parB</td>
<td>471bp</td>
<td>MX 1</td>
</tr>
<tr>
<td>HI1 RV</td>
<td>tgcgctttcactctgtagta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HI2 FW</td>
<td>ttctctgagtcactctgttaac</td>
<td>iterons</td>
<td>644bp</td>
<td>MX 1</td>
</tr>
<tr>
<td>HI2 RV</td>
<td>ggctcactacgtgttcactct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I1 FW</td>
<td>cgaagccgcaacggcagaa</td>
<td>RNAI</td>
<td>139bp</td>
<td>MX 1</td>
</tr>
<tr>
<td>I1 RV</td>
<td>tcgtgatcgcacagtagtgc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X FW</td>
<td>aaccttagagcctttaagttgctgat</td>
<td>ori λ</td>
<td>376bp</td>
<td>MX 2</td>
</tr>
<tr>
<td>X RV</td>
<td>tgcagctcaatttttatctacgttttagc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L/M FW</td>
<td>ggatgaaaacatacagcctagtaag</td>
<td>repA,B,C</td>
<td>785bp</td>
<td>MX 2</td>
</tr>
<tr>
<td>L/M RV</td>
<td>ttcacaggggcttcttttgag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N FW</td>
<td>gtcctagagctttaacagcag</td>
<td>repA</td>
<td>559bp</td>
<td>MX 2</td>
</tr>
<tr>
<td>N RV</td>
<td>gttcacaactgcacagttc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIA FW</td>
<td>ccatgctgatcttagagaaagttg</td>
<td>iterons</td>
<td>462bp</td>
<td>MX 3</td>
</tr>
<tr>
<td>FIA RV</td>
<td>gctatccctacttgcttcaggcag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIB FW</td>
<td>ggagttctgacacagatttcttcg</td>
<td>repA</td>
<td>702bp</td>
<td>MX 3</td>
</tr>
<tr>
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<td>SX 3</td>
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11 K & B/O FW, due to the high level of homology between the K and B/O replicons the same forward primer was used in both K and B/O simplex PCRs.
### Table 2.7: Extended antiobigram, PCR, sequence analysis and molecular typing of ‘cefepimase’ producing S. Typhimurium

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Origin</th>
<th>Resistance profile*</th>
<th>β-lactamases</th>
<th>Class I integrons</th>
<th>2kb VR</th>
<th>0.8kb VR</th>
<th>Plasmid Profile (kb)</th>
<th>XbaI PFGE</th>
<th>BlnI PFGE</th>
<th>MLST type</th>
<th>Sequence</th>
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<td>OXA-1, TEM-1</td>
<td>2 Class I integrons</td>
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<td>8.8, 16.5, 39</td>
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<td>blaOXA₄, aadA₁, aadB</td>
<td>8.8, 16.5, 39, 105</td>
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</tr>
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* A= Ampicillin; C = Chloramphenicol; S = Streptomycin; Su = Sulphonamides; T = Tetracycline; Cpd = Cefpodoxime; Mh = Minocycline; Cpo = Cefpirome; Cxm = Cefuroxime; Rif = Rifampicin; Na= Nalidixic Acid; W = Trimethoprim; K = Kanamycin; Cn = Gentamicin)

VR= Variable region of class 1 integron
2.3.10 Isoelectric focusing (IEF)
Enzyme extractions were carried out on all isolates using a freeze thaw procedure. Enzyme extracts were loaded onto Amersham Biosciences 1mm Polyacrylamide Gel (pH 3.5-9.5). Focused gels were overlaid with 54M filter paper soaked in nitrocefin (0.5mg/ml). To determine the isoelectric point (pI) of the enzymes a standard curve was constructed using control β-Lactamase extracts of TEM-3 (pI 6.3), TEM-4 (pI 5.9), TEM-12 (pI 5.25), SHV-3 (pI 7.0) and SHV-5 (pI 8.2) (Figure 2.1).

The detailed IEF protocol is documented under ‘Protocols-Protocol # 9’ on attached CD disk.
Figure 2.1  Standard curve used in determination of the pI of β-lactamase enzymes isolated from ‘cefepimase’ producing S. Typhimurium - pI versus distance migrated from anode, generated using the known pI’s of the control enzymes TEM-12, SHV-3 and SHV-5.

The equation of the line (m or y = (y₂ - y₁) / (x₂ - x₁)) was used to calculate the isoelectric point (pI) of unknown β-lactamases harboured by ‘cefepimase’ S. Typhimurium

\[ y = -20.286x + 176.27 \]

\[ R^2 = 0.9911 \]
2.3.11 Cloning and sequencing of resistance plasmid

pFEP39 was transferred into Top10 electrocompetent cells by electroporation for propagation. pFEP39 was digested with BamH1/Xba1 and fragments cloned into the corresponding sites of the kanamycin resistance pBK-CMV expression vector (Stratagene) followed by transformation into Top 10 E. coli cells. Transformants were selected on LB agar containing cefepime (4µg/ml) and kanamycin (25µg/ml) containing LB agar. A number of clones were selected for further study including the cefepime resistant clone eNUI-2009. Plasmid pFEP39-1dr was extracted from eNUI-2009 and a c. 5kb insert was sequenced using T3/T7 primers, flanking the multiple cloning site of the pBK-CMV expression vector.

2.3.12 Site directed mutagenesis (SDM)

The role of a triplet ‘GGG’ residue upstream of the blaOXA-1 β-lactamase in the cloned segment ‘1dr’ was investigated by site directed mutagenesis. This was carried out by developing primers (del1214 and del1214-1216as) containing the desired mutation (deletion of ‘GGG’ triplet at nucleotide position 1214-1216 of pFEP39-1dr) for incorporation into pFEP39-1dr (Table 2.3). The QuikChange XL site directed mutagenesis (Stratagene) kit was utilised for this investigation. Primers were developed to amplify the sequence of interest (SDM-Fw and SDM-Rv) with subsequent sequencing of the amplicon to confirm ‘GGG’ deletion.

The QuikChange XL site directed mutagenesis protocol is documented under ‘Protocols-Protocol # 10’ on attached CD disk.

2.3.13 Sequencing

Sequencing of all amplicons was performed on both strands using primers homologous to those used for PCR on ABI 3730 capillary sequencers by Sequiserve, Vaterstetten, Germany.

The PCR product purification protocol is documented under ‘Protocols- Protocol # 2’ on attached CD disk.
2.3.14 Nucleotide sequence accession number

The nucleotide sequence data reported in this paper is logged in GenBank nucleotide sequence database under accession no. GU119958.

2.4 Results:

Results are summarised in Table 2.7, 2.8 and 2.9.

Seventeen isolates (all S. Typhimurium) had cefepime MICs of 8 to 32μg/ml falling to 0.064 to 0.25 μg/ml in the presence of clavulanic acid, cefotaxime MICs of 0.25 to 2 μg/ml falling to 0.064 to 0.125 μg/ml with clavulanic acid and ceftazidime MICs of 0.5μg/ml (Table 2.8). Four different antimicrobial resistance profiles were observed with all isolates resistant to ampicillin, streptomycin, sulphonamides, cefpodoxime, cefpirome, cefuroxime and rifampicin. The combination disk method using cefpodoxime and cefpodoxime plus clavulanic acid failed to confirm ESBL production in any of the “cefepimase” producers. Isoelectric focusing identified 2 β-lactamases with isoelectric points (pIs) of 5.2 and 7.2. All ‘cefepimase’ producers harboured blaTEM-1 and blaOXA-1, (as confirmed by PCR and amplicon sequencing) and were negative for all other β-lactamase genes tested. All 19 isolates harboured a Class I integron of 2kb. Fifteen isolates also harboured a Class I integron of 800bp and all were negative for Class II integrons (Table 2.7).

All isolates with blaOXA-1 also were positive for ISCR1. PFGE analysis gave 14 and 9 distinguishable DNA banding patterns with XbaI and BlnI respectively-Figures 2.2 and 2.3). All African ESBL producers assigned to ST313 and the Irish ESBL producer assigned to ST19 (Table 2.7). Therefore, XbaI PFGE results suggest a level of genetic diversity (75-100% similarity on PFGE dendogram, see Figure 3.3) within ST313 of S. Typhimurium.

VNTR analysis generated 13 allele profiles from the 19 ‘cefepimase’ producing isolates (Table 2.9). Thirteen different plasmid profiles of 2 to 5 plasmids and with plasmids ranging from 7 to 135kb were recognized (Table 2.7). Southern hybridization of plasmid gels revealed an OXA Group 3 like gene on a plasmid
of a c.39kb (pFEP39) in all ‘cefepimase’ producers. Following liquid mating at 25°C the transfer of pFEP39 was associated with transfer of the ‘cefepimase’ phenotype to S. Dublin JE066 (Table 2.8). On PBRT analysis pFEP39 was a multi replicon plasmid of IncW and HI2 type. Inc W and HI2 PCR products were confirmed by sequencing. All ‘cefepimase’ isolates containing the 39kb plasmid were positive on PBRT for IncW and HI2 replicons. IncFIIIs plasmids
(Salmonella virulence plasmid) were also present in all S. Typhimurium. Other Inc groups identified were IncFIA in 6 isolates, IncK in 3 with IncI1, IncX and IncA/C identified in 3 separate isolates. ClustalW2 alignment revealed 100% identity to published Inc W and IncHI2 sequences.

Shotgun cloning of pFEP39 into the XbaI BamHI restriction site of expression vector pBK-CMV resulted in recombinant plasmids with inserts of circa 5kb, 7.5kb, 9kb and 10kb. The plasmid containing the insert of c.5kb (pFEP39-1dr) was associated with the “cefepimase” phenotype when transformed into Top10 electrocompetent E. coli cells. MICs of transformants are detailed in Table 2.8. Sequencing of a 4.937kb insert from recombinant plasmid pFEP39-1dr confirmed the β-lactamase blaOXA-1/30 as the only β-lactamase present in the transformed Top10 cells. The blaOXA-1/30 was located on a BamHI restriction fragment of c. 5 kb (pFEP39-1dr) from all pFEP39 plasmids isolated from ‘cefepimase’ producers (Figure 2.4).

The cloned sequence revealed an unusual Class 1 integron promoter combination upstream of blaOXA-1 (Figure 2.5). The P\textsubscript{ant}/P\textsubscript{c} promoter combination identified in insert ‘1dr’ was TGGACA\textsubscript{17}TAAGCT (-35/-10 sequence), downstream of which there was a P\textsubscript{2} promoter combination of TTGTTA\textsubscript{17}TACAGT (-35/-10 sequence). Deletion of the ‘GGG’ triplet immediately upstream of the -10 region of P\textsubscript{2} by site directed mutagenesis (SDM) resulted in loss of the ‘cefepimase’ phenotype (Figure 2.6). This mutated plasmid was named Δ\textsuperscript{1}pFEP39-1dr.
DNA sequencing results and associated analyses/alignments etc. related to the “cefepimase” producers is documented on attached CD under the following designations:

- Chapter 2 DNA sequencing results- TEM & OXA
- Chapter 2 DNA sequencing results- MLST
- Chapter 2 DNA sequencing results- PBRT
- Chapter 2 DNA sequencing results- pFEP39-1dr
Table 2.8 Minimum Inhibitory Concentration’s (MICs) of strains (μg/ml)

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<th>Isolate No.</th>
<th>MIC (μg/ml)</th>
<th>β-lactamases present</th>
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<td>CT/CTL</td>
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<tr>
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<td>1.5/0.125</td>
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<tr>
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<td>0.5/0.25</td>
<td>2.0/0.125</td>
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<td>0.5/0.38</td>
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</tr>
<tr>
<td>27</td>
<td>0.5/0.25</td>
<td>1/0.094</td>
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<td>32</td>
<td>0.5/0.25</td>
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<td>&lt;0.5/0.064</td>
<td>&lt;0.25/&lt;0.016</td>
</tr>
<tr>
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<td>XL10</td>
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<td>&lt;0.25/&lt;0.016</td>
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</table>

* Transconjugant Salmonella Dublin with pFEP39 plasmid

**Top10 electrocompetent cells with recombinant plasmid pFEP39-1dr conferring phenotype

***XL10 ultra competent cells harboring pFEP39-1dr mutant (Δ') with ‘GGG’ triplet deleted immediately upstream of P₂ promoter
### Table 2.9  MLVA results of ‘cefepimase’ producing *S. Typhimurium*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>STRR9-8bp</th>
<th>STTR5-bp</th>
<th>STTR6-bp</th>
<th>STTR10-bp</th>
<th>STTR3-bp</th>
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<tr>
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</tbody>
</table>

* Larsson *et al* 2009,[43] as per revised MLVA nomenclature
Figure 2.2 *Bln*I PFGE cluster analysis of ‘cefpimase’ producing *S. Typhimurium*
Figure 2.3 *XbaI* PFGE cluster analysis of ‘cefepimase’ producing *S. Typhimurium*
Figure 2.4  *Bam*H1 RFLP and DIG-Labelled OXA-1 probing of *Bam*H1 Southern blots of pFEP39

Group A = 10
Group B = 4
Group C = 2
Group D = 1
Group E = 1
Group F = 1
**Figure 2.5**: Schematic representation of the DNA acquired by pBK-CMV to compose pFEP39-1dr
Figure 2.6: Comparison of upstream sequences of P₂ promoter (deleted ‘GGG’ observed in mutant Δ¹ pFEP39-1dr versus control non mutated plasmid pFEP39)

Δ¹ pFEP39-1dr  
GGCGCCCAATGCCTGACGATGCGTGGAGACCGAACCTTGCGCTCGTTCGCCAGCCAGGA 60
pFEPControl    
GGCGCCCAATGCCTGACGATGCGTGGAGACCGAACCTTGCGCTCGTTCGCCAGCCAGGA 60

******************************************************************************

Δ¹ pFEP39-1dr  
CAGAAATGCTCGACTTCCGCTGCTGCTGCCAAGGTTGGCGTGAAGCACACCTGGGAAC 120
pFEPControl    
CAGAAATGCTCGACTTCCGCTGCTGCTGCCAAGGTTGGCGTGAAGCACACCTGGGAAC 120

******************************************************************************

Δ¹ pFEP39-1dr  
GATGAAGGCACGAACCAATGGAGATGCTGTTTCTGCTGTTAAGCTGTAATGCAAGTA 180
pFEPControl    
GATGAAGGCACGAACCAATGGAGATGCTGTTTCTGCTGTTAAGCTGTAATGCAAGTA 180

******************************************************************************

Δ¹ pFEP39-1dr  
GCCTGATGCACGTCAAGCAACCACGGAGGTGGCTGGCTGTAACGGGGCC 240
pFEPControl    
GCCTGATGCACGTCAAGCAACCACGGAGGTGGCTGGCTGTAACGGGGCC 240

******************************************************************************

Δ¹ pFEP39-1dr  
AGTGCCCCTTCTATGGCTTGTTATGACTGTTTTTTTG------TACAGTCTATGCTCGGC 297
pFEPControl    
AGTGCCCCTTCTATGGCTTGTTATGACTGTTTTTTTGAGGGTACAGTCTATGCTCGGC 300

******************************************************************************

Δ¹ pFEP39-1dr  
ATCCAAGCAGCAAGCAGTCCGGCCTGTTGTTATGTTATGGAGCAGCAAGCA 357
pFEPControl    
ATCCAAGCAGCAAGCAGTCCGGCCTGTTGTTATGTTATGGAGCAGCAAGCA 357

******************************************************************************

Δ¹ pFEP39-1dr  
TGTTAGCAGCAGGCAATGCCTGGCGTGTTATGTTATGGAGCAGCAAGCA 379
pFEPControl    
TGTTAGCAGCAGGCAATGCCTGGCGTGTTATGTTATGGAGCAGCAAGCA 382

******************************************************************************
2.5 Discussion:
The main focus of this work was to identify the genetic basis for the novel β-lactamase phenotype conferring marked elevation of the cefepime MIC (relative to cefotaxime or ceftazidime) associated with significant inhibition by clavulanic acid. Other than the single isolate associated with travel to Andorra, we have not observed this phenotype in other *S. enterica* isolates in the database of National Salmonella Reference Laboratory of Ireland (including the 9733 *S. Typhimurium* isolates received at NSRL since its establishment in 2000). We are not aware of prior reports of this phenotype from elsewhere.

Members of the CTX-M family of β-lactamases have been associated with high-level resistance to cefepime together with high level resistance to cefotaxime [250]. However, our isolates did not exhibit high cefotaxime MIC’s and were repeatedly negative for known *bla*<sub>CTX-M</sub> genes (*Table 2.7* and *Table 2.8*). Hyperproduction of SHV-5 has also been associated with elevated cefepime MICs but in association with high level resistance to cefotaxime (MIC = 128 μg/ml) and ceftazidime (MIC = 128 μg/ml) [251] and again *bla*<sub>SHV</sub> genes were not detected in this collection of isolates. The β-lactamase genes *bla*<sub>TEM-1</sub> and *bla*<sub>OXA-1/30</sub> were readily identified on PCR and this was consistent with the detection of two β-lactamase enzymes (pI 5.2 corresponding to TEM-1 and 7.2 corresponding to OXA-1) on isoelectric focusing. The OXA family of β-lactamases is extremely diverse and can be divided into 5 groups based on amino acid sequence homology [226]. Cefepime resistance together with moderate resistance to cefotaxime and susceptibility to ceftazidime has been reported in association with Group III OXA which comprises OXA-1, 4, 30 and 31, however in all such reports inhibition with clavulanic acid was weak or absent [124, 252]. OXA-9, 18, 45 and 53 are the only OXA enzymes reported to be significantly inhibited by clavulanic acid [104]. Our initial hypothesis was that some other uncommon β-lactamase may be present to account for the phenotype. For example Potron et al. [236] has described a similar phenotype in *Acinetobacter baumanii* due to production of *bla*<sub>RTG-4</sub>. However, neither *bla*<sub>RTG-4</sub> nor any other *bla* genes were identified and the phenotype was transferred to *S. Dublin* and to Top10 *E. coli* cells in association with plasmid pFEP39 on which the only *bla* gene detected...
was bla\(_{OXA1/30}\). A 39 kb IncHI2/ IncW multireplicon plasmid was present in all isolates studied. The phenotype was also expressed by Top10 \(E.\ coli\) cells transformed with a recombinant plasmid (eNUI-2009) containing a c. 5 kb insert (pFEP39-1dr) from pFEP39 including \(bla_{OXA-1/30}\) and an activated \(P_{ant}/P_c–P_2\) Class I integron promoter combination upstream of \(bla_{OXA-1/30}\) (Figure 2.5).

XL10 gold cells containing the pFEP39-1dr have cefepime & cefepime/ clavulanic acid MICs of 16\(\mu\)g/ml and 0.19\(\mu\)g/ml respectively compared to 0.06\(\mu\)g/ml and 0.06\(\mu\)g/ml respectively for XL10 native cells. The \(P_{ant}/P_2\) promoter has been associated with a dramatic increase in the level of expression of the proximal gene in the integron cassette array [253-255] e.g. \(aadA1\) expression increased by approximately 20 fold [256]. However, to the best of our knowledge this is the first report of this combination resulting in increased expression of a \(\beta\)-lactamase gene. Similarly, \(\beta\)-lactamase hyperproduction was highlighted by Brízio et al. when a hyper producing IMP-5 spreading among clinical isolates of \(P.\ aeruginosa\) (due a Class 1 integron with a point mutation in the \(P_1\) promoter -35 hexamer) was identified [257]. A similar phenomenon was also observed in a \(bla_{TEM-1}\) hyper-producing \(Shigella\ flexneri\) isolate from Hong Kong resulting from a point mutation in the -10 hexamer of the pribnow box [258]. In the “cefepimase” collection, \(P_2\) had been activated by the presence of a triplet ‘GGG’ immediately upstream of the -10 signal. Targeted deletion of the ‘GGG’ triplet immediately upstream of \(P_2\) by Site Directed Mutagenesis (SDM) (to give plasmid \(\Delta\)\(P_{ant}/P_3\)FEP39-1dr) resulted in a 32 fold decrease in cefepime MIC (to 0.5\(\mu\)g/ml and reduced further to 0.03\(\mu\)g/ml in the presence of clavulanic acid).

There is therefore persuasive evidence that the phenotype observed is related to plasmid encoded high level expression of \(bla_{OXA-1/30}\). Kinetic studies are planned to further define the enzyme substrate and inhibition profile.

In association with \(bla_{OXA-1/30}\) we also identified IS\(CR1\), an element with which various \(bla\) genes, including \(bla_{CTX-M}, bla_{CMY}, bla_{DHA-1}, bla_{VEB}, bla_{MOX-1}, bla_{PER-3}\), have previously been associated [190]. Such elements have the potential to act as promoters for certain \(\beta\)-lactamases [190, 259-264] and are believed in certain cases to increase expression or hyper production of the gene [264-266]. This was not the case in our collection as in our recombinant plasmid containing insert ‘1dr’, \(bla_{OXA-1}\) was not cloned in conjunction with IS\(CR1\), therefore confirming
that the phenotype is not resultant of hyper production due to this insertion element.

Although the number of isolates is relatively small the chromosomal diversity of the collection (heterogeneous on PFGE and MLVA typing) suggests that the plasmid has disseminated extensively within S. Typhimurium. Multi replicon plasmids have been reported in Salmonella previously including IncH plasmids carrying ESBL genes [120, 267]. However, to the best of our knowledge this is the first report of a novel multi replicon plasmid with the Inc HI2 /IncW replicon combination. IncW plasmids exhibit a broad host range including S. Typhimurium [268].

All isolates harbored a c. 39kb plasmid (pFEP39). The purified plasmid extract of pFEP39 was replicon Inc HI2 and Inc W positive upon PBRT analysis. The pFEP39 plasmid is therefore a multi-replicon Inc HI2/ Inc W plasmid. The Inc HI2/ Inc W pFEP39 plasmid was transferable by conjunction with the ‘cefepimase’ phenotype from all donor isolates into recipient E. coli J53 or S. Dublin JE066. All IncH12 and Inc W amplicons exhibited 100% sequence identity. Furthermore, the size of IncH12 plasmids that have been previously sequenced is generally >200 kb (A. Carattoli, personal communication). Therefore, it may be hypothesised that the replicon scaffold is mostly IncW that have been fused to an IncHI2 replicon resolving the structure in a way that it maintained the replicon from the HI2 (A. Carattoli, personal discussion and communication).

BamHI restriction profiles of pFEP39 isolated from each transconjugant yielded 6 BamHI restriction profiles. The blaoxa-1/30 gene was located on a BamHI restriction fragment of c. 5 kb (pFEP39-1dr) from all pFEP39 plasmids isolated from ‘cefepimase’ producers. All profiles were similar in their restriction pattern by BamHI RFLP, however were not identical. Upon BamHI RFLP analysis the size of each plasmid conferring the ‘cefepimase’ phenotype the size of each may not be exactly 39kb. It is important to point out that the method of plasmid analysis used for sizing plasmids for this portion of my PhD research was alkaline lysis. This method is prone to errors in sizing of plasmids (refer to
chapter 4 for further information and discussion on this topic). Therefore, it is possible that there could be subtle variation in the size of the plasmid conferring the ‘cefepimase’ phenotype in each donor. All pFEP39 plasmids were PCR positive for \( \text{bla}_{\text{OXA}-1} \), \( \text{bla}_{\text{TEM}-1} \), IncH12 and IncW. All sequences were 100% identical between all pFEP39 plasmids. This further supports the similarity of all pFEP39 plasmids (from each donor).

It would be interesting to further characterize each pFEP39 plasmid from each transconjugant. A method to further characterize these plasmids is pMLST – plasmid MLST. The choice of genetic markers to be used during pMLST includes those encoding maintenance and replication plasmid functions. In addition, these genetic markers are relatively well conserved but also show significant nucleotide differences that are potentially useful in subtyping plasmids. pMLST may be applied as a second tier of analysis to gain more knowledge on the diversity amongst the nineteen pFEP39 IncH12/IncW multi-replicon plasmids carrying \( \text{bla}_{\text{OXA}-1} \) (with the promoter combination) presented in this thesis. The information on pFEP39 in this thesis can hypothesize each plasmid may be derived from a single IncHI2 ancestor plasmid that was subjected to different rearrangement events. The diversity seen in the \textit{BamH}1 RFLP profiles of the pFEP39 IncH12/IncW plasmids suggests intra-plasmid evolution of these elements by multiple recombinatorial events.

pFEP39 is present in both the multi drug resistant invasive \textit{S. Typhimurium} ST313 clonal group and in a single \textit{S. Typhimurium} ST19 clone recovered from an Irish patient following travel to Andorra. ST19 is the most commonly identified Typhimurium ST and is recovered globally, with ST313 being a single locus variant (SLV), specifically localised to the sub Saharan region of Africa [269]. Kingsley \textit{et al.} highlighted that ST313 and ST19 \textit{S. Typhimurium} are circulating simultaneously in Kenya, but ST313 is the only ST identified in isolates recovered from Malawi, it is likely that ST313 has begun to replace ST19 as the predominant clone in this region [269]. Co-circulation of two STs present the opportunity for genetic exchange and could potentially explain the dissemination of the pFEP39 plasmid among the two clonal groups. The presence of the phenotype was checked in a collection of 10,500 \textit{S. Typhimurium} isolates
collected from Irish patients between 2000 and 2011, from our laboratory. The ST19 clone was the isolate that exhibited this phenotype and was associated with travel to Andorra. This highlights the problem of the air travel generation. Air travel allows for rapid dissemination of new disease variants globally and thus requires better communication between public health authorities in reporting new emerging diseases so that governments can better prepare for and monitor the spread of diseases globally.

As the collection of isolates is predominantly from Kenya this resistance phenomenon may be relatively restricted to parts of sub-Saharan Africa. However, it is important to note that recommendations for confirmation of ESBL production that advocate the use of cefotaxime and ceftazidime or cefpodoxime with and without clavulanic acid may result in failure to detect such isolates as ESBL producers [203, 219, 270, 271]. The cefepime-clavulanic acid ESBL Etest strip is generally reserved for screening for ESBL production in isolates that co-produce AmpC β-lactamases [203]. Stürenburg et al. reported that the cefepime-clavulanic acid ESBL Etest strip was more sensitive than the cefotaxime-clavulanic acid and ceftazidime-clavulanic acid ESBL Etest strips for detection of ESBL production in a collection of ESBL producing Enterobacteriaceae [205]. In addition, it is important to note that the recent CLSI M20 standard states that when using the updated M21 guidelines (which include revised cephalosporin MIC’s) it is no longer necessary to carry out routine ESBL confirmation before reporting cephalosporin MIC’s [194]. This is also the stance taken in the recent guidelines published by EUCAST [193]. Therefore, according to CLSI and EUCAST guidelines, confirmation of ESBL production utilising clavulanic acid synergy with 3rd generation cephalosporins is no longer required prior to administration of antimicrobial therapy, however it is imperative that ESBL production continues to be screened for and confirmed for epidemiological and infection control purposes.

3.6 Conclusion
The occurrence of this phenotype among genetically diverse *Salmonella* Typhimurium from Kenya, Ireland and Malawi highlight the potential for
widespread dissemination of such a resistance threat. However, to the best of our knowledge, this is the first study to report the observation of an ESBL phenotype resulting from expression of \textit{bla}_{\text{OXA-1}} due to the simultaneous presence of a Class 1 integron \textit{P}_{\text{ant}}/\textit{P}_{\text{c}} promoter accompanied by an activated \textit{P}_{2} promoter. Further studies are required to determine the extent of geographic dissemination of this resistance phenomenon in \textit{Salmonella enterica} and to determine if the plasmid has disseminated into other \textit{Enterobacteriaceae}. 
Chapter 3

First report of extended-spectrum \( \beta \)-lactamase producing Salmonella Kentucky isolated from poultry in Ireland
3.1 Abstract:

This work reports the emergence of plasmid mediated broad spectrum cephalosporin resistance in S. Kentucky isolated from poultry specimens in Ireland. In February 2009 we reported the first occurrence of both Extended-Spectrum β-lactamase producing and AmpC producing Salmonella isolated from poultry products in Ireland. Further analysis revealed the ESBL, SHV-12, to be present in 4 of 7 S. Kentucky with the plasmid-mediated AmpC, CMY-2, detected in 3 of 7 S.Kentucky. A previous report of a similar phenomenon with S.Virchow from poultry products in France, Belgium, The Netherlands and Ireland reinforces the public health significance of this finding [4, 152, 168]. The occurrence of such plasmid-mediated antimicrobial resistance determinants highlights the potential for transfer of such determinants through the food chain and into humans.

3.2 Introduction:

The spread of aetiological agents of infection via the food chain persists as one of the most widespread public health problems not only in the developing world but also in the developed world. The vast majority (86%) of human cases of salmonellosis are documented to be attributable to foodborne transmission [272]. Salmonella are an important cause of human food borne infection worldwide. Although antimicrobials are not usually recommended in cases of salmonella enterocolitis, they become an essential part of management if the infection spreads beyond the gastrointestinal tract. Complications of invasive disease include meningitis, arthritis, and deep-seated abscesses and are more common in infants, the elderly and immuno-compromised.

β-lactamase enzymes are the predominant method of resistance to β-lactam antimicrobial agents among the Enterobacteriaceae. Refer to Chapter 1 section 1.3.1 for the definition of β-lactamase and extended-spectrum β-lactamases (ESBLs) past and present. Briefly, bacteria possessing an ESBL are resistant to the 3rd and 4th generation cephalosporins, however bacteria possessing the ESBL
gene remain susceptible to the cephamycin’s. Plasmid-mediated AmpC β-lactamase enzymes confer resistance to the 3rd generation cephalosporins and the cephamycin’s, in addition plasmid-mediated AmpC β-lactamase enzymes are resistant to inhibition by β-lactamase inhibitors, such as clavulanic acid, and only weakly hydrolyse 4th generation cephalosporins such as cefepime. Salmonellae do not possess a chromosomal AmpC β-lactamase and therefore its presence is due to acquisition of mobile genetic elements coding these enzymes [273]. The first report of AmpC type β-lactamase (blaCMY-2) presence in Salmonella was described in 1988 [148]; thereafter the prevalence has dramatically increased [122, 274].

It is hypothesised that the eradication of the poultry specific serovars Pullorum and Gallinarum have exposed an ecological niche for other non-host specific serovars such as S. Enteritidis, S. Kentucky and S. Typhimurium [272]. Of further concern is the emergence and dissemination of antimicrobial resistance determinants, such as blaCMY-2, among Salmonella strains e.g. S. Newport from cattle in the USA, S. Heidelberg from poultry in Canada [275]. In addition, interspecies transmission of plasmids harbouring blaCMY-2 has been reported [276]. In this study Poppe and colleagues reported that an IncA/C plasmid harbouring blaCMY-2 was transferred from an E.coli strain to a S. Newport within the same chicken. IncA/C plasmids of animal origin have been identified in humans infected with S. Newport and S. Typhimurium [277, 278]. A recent study by Subbiah and colleagues highlighted that even though IncA/C plasmids are particularly successful at dissemination within Salmonella and other Enterobacteriaceae, IncA/C plasmids impose a fitness cost to the host bacterium in the absence of a selecting agent [279]. The use of β-lactams in the animal industry is hypothesised to select for these plasmids and aid in their dissemination. This was illustrated by Subbiah and colleagues both in-vitro and in-vivo through the use of ceftiofur as the selecting agent [279]. The withdrawal of ceftiofur in these studies resulted in the same strains of Salmonella persisting, however the IncA/C plasmids were eventually lost from the particular population [279]. A recent study by Martin and colleagues has reported that blaCMY-2 harbouring plasmids are quite promiscuous with IncA/C, IncI1, IncK and IncFIB
shown to harbour and aid in the dissemination of $bla_{\text{CMY-2}}$ among $S. \text{enterica}$ and $E. \text{coli}$ [280].

Recently, a Salmonella Typhi isolate possessing an IncA/C plasmid harbouring $bla_{\text{CMY-2}}$ was identified from the blood culture of a child in India [281]. This is a cause for concern as the dissemination of extended-spectrum cephalosporin resistance among Salmonella Typhi further limits the therapeutic options available for the treatment of Typhoid fever. The authors hypothesised that the IncA/C harbouring $bla_{\text{CMY-2}}$ was acquired from a multidrug resistant member of the Enterobacteriaceae [281].

Prior to 2002 $S. \text{Kentucky}$ was mainly isolated from poultry, seafood and cattle [282] and was considered not a major contributor to the total number of cases of gastroenteritis caused by Salmonella serovars [29]. However, since 2002 $S. \text{Kentucky}$ has increased in prevalence as a cause of gastroenteritis in humans [283] [282] [284]. $S. \text{Kentucky}$ is now ranked in the top ten serovars isolated from humans in Europe [282]. Compounding this is the emergence of multidrug resistant $S. \text{Kentucky}$ belonging to the ST198 lineage. The ST198 lineage of $S. \text{Kentucky}$ is resistant to ciprofloxacin. A recent study by Hello and colleagues highlighted that in the study group of France, England, Wales and Denmark the number of cases of ciprofloxacin resistant $S. \text{Kentucky}$ increased from 3 in 2002 to 174 in 2008 [283]. A study by Bonalli and colleagues highlighted that $S. \text{Kentucky}$ is now ranked 8-10 of the most common serovars isolated from humans in Switzerland, whereas it was not in the top 20 in 2000. Only one of 106 $S. \text{Kentucky}$ strains isolated in Switzerland from 2004-2009 harboured an ESBL although 58% were resistant to ciprofloxacin [282]. In contrast, in the USA, of 679 strains of $S. \text{Kentucky}$ reported to the CDC from 2002-2008, none were resistant to ciprofloxacin [283].

Foreign travel to Africa combined with the import of spices and raw vegetables from areas of endemicity may be contributing to the dissemination of ciprofloxacin resistant $S. \text{Kentucky}$ across Europe [283]. The spread of ciprofloxacin resistant $S. \text{Kentucky}$ within countries such as Egypt, Morocco and Nigeria is linked to intensive poultry farming, the use of fluroquinolones in
farming, and consumption of contaminated shellfish (due to water contaminated through poultry or humans) [283]. Individuals infected with multi drug resistant S. Kentucky strains are more likely to be hospitalised than those infected with pan susceptible strains of S. Kentucky [283].

3.3 Materials and methods:
A detailed list of the materials, methods and equipment used for this work are documented under ‘Chapter 3 Materials, and equipment’ on attached CD disk

3.3.1 Bacterial strains
Between January 2000 and September 2008 the Irish National Salmonella Reference Laboratory (INSRL) received and analysed 925 *Salmonella enterica* subsp. *enterica* serovar Kentucky of both human and animal origin, with all being susceptible to cephalosporins. Between October 2008 and February 2009, 115 *S*. Kentucky isolates were received. Seven of the 115 *S*. Kentucky strains exhibited resistance to the cephalosporins. In October 2008, the first ESBL producing *S*. Kentucky (08-1060) was isolated with a further 6 (08-1259, 09-031, 09-060, 09-061, 09-0135 and 09-0171) isolated between October 2008 and February 2009. All were from poultry. *(Table 3.1, Figure 3.1).*
Table 3.1  
Summary of β-lactamase producing *S*. Kentucky isolates included in this thesis

<table>
<thead>
<tr>
<th>Farm</th>
<th>Isolate</th>
<th>Date isolated</th>
<th>Specimen</th>
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</thead>
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<td>07/10/2008</td>
<td>Whole chicken</td>
</tr>
<tr>
<td>F2</td>
<td>08-1259</td>
<td>25/11/2008</td>
<td>Chicken neck skin</td>
</tr>
<tr>
<td>F3</td>
<td>09-031</td>
<td>16/01/2009</td>
<td>Poultry broiler dust</td>
</tr>
<tr>
<td>F3</td>
<td>09-060</td>
<td>03/02/2009</td>
<td>Whole chicken</td>
</tr>
<tr>
<td>F4</td>
<td>09-061</td>
<td>03/02/2009</td>
<td>Whole chicken</td>
</tr>
<tr>
<td>F5</td>
<td>09-135</td>
<td>18/02/2009</td>
<td>Poultry broiler dust</td>
</tr>
<tr>
<td>Unknown</td>
<td>09-171</td>
<td>06/03/2009</td>
<td>Whole chicken</td>
</tr>
</tbody>
</table>
Figure 3.1 Location of poultry farms in Ireland from which β-lactamase producing *S. Kentucky* were isolated

<table>
<thead>
<tr>
<th>Isolate #</th>
<th>Farm #</th>
</tr>
</thead>
<tbody>
<tr>
<td>08-1060</td>
<td>F1</td>
</tr>
<tr>
<td>08-1259</td>
<td>F2</td>
</tr>
<tr>
<td>09-031</td>
<td>F3</td>
</tr>
<tr>
<td>09-060</td>
<td>F3</td>
</tr>
<tr>
<td>09-061</td>
<td>F4</td>
</tr>
<tr>
<td>09-135</td>
<td>F5</td>
</tr>
<tr>
<td>09-171</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
3.3.2 Serotyping:
Upon receipt of *Salmonella* isolates serotyping was carried out to confirm the serovar. Serological analysis of the somatic or cell wall (O) antigens and flagellar (H) antigens of *Salmonella* was carried out on all isolates in accordance with the Kaufmann-White classification scheme, Pasteur Institute, Paris using commercially available antisera. A pure culture of the isolate was used for O antigen detection and a broth growth from the bottom of an agar slope was utilised for H antigen detection. Positive and negative control antiserum was used during analysis.

3.3.3 Antimicrobial susceptibility testing:
All isolates were tested for susceptibility to the following antimicrobial agents in accordance with CLSI disk diffusion methods [219]: ampicillin (10μg), chloramphenicol (30μg), streptomycin (10μg), sulphonamides (300μg), tetracycline (30μg), trimethoprim (5μg), nalidixic acid (30μg), ciprofloxacin (5μg), gentamicin (10μg), kanamycin (30μg), minocycline (30μg) and cefoxitin (30μg). All antimicrobial disks were received from Oxoid, Basingstoke, U.K.

3.3.4 Confirmation of ESBL production
Isolates were screened for ESBL production in accordance with Clinical Laboratory Standards Institute (CLSI) disk diffusion methods using cefpodoxime (10μg), cefotaxime (30μg) and ceftazidime (30μg) [219]. Suspect ESBL production was confirmed by the combination disk method of CLSI using cefpodoxime (30μg), and cefpodoxime plus clavulanic acid (10μg/1μg) and by the ESBL Etest method using ceftazidime/ceftazidime plus clavulanic acid (TZ/TZL); cefotaxime/cefotaxime plus clavulanic acid (CT/CTL); and cefepime/cefepeime plus clavulanic acid (PM/PML) Etest strips in accordance with the manufacturers’ instructions (AB Biodisk, Solna, Sweden).

3.3.5 DNA extraction of bacterial isolates:
DNA extraction was carried out on all bacterial isolates using the QIAamp DNA Mini Kit (Qiagen Inc. Valencia Calif) in accordance with the manufacturer’s instructions. DNA extracts were stored prior to molecular work at -20°C. The
Chapter 3

DNA extraction protocol is documented under ‘Protocols-Protocol # 1’ on attached CD disk.

3.3.6 Polymerase Chain Reaction (PCR):
PCR primers and annealing temperatures used are documented in ‘Chapter 3-PCR Primers and amplification conditions’ on attached CD disk.

The PCR was carried out on all isolates using specific primers for \(blat_{TEM}\), \(blashv\) and \(bla_{CTX-M}\) Groups 1, 2, 8, 9 & 25, plasmid-mediated \(bla_{ampC}\), Salmonella Genomic Island 1 (SGI1) and Class 1 Integrons. Absence of inhibitors of PCR was confirmed in all DNA preparations by amplification of the 16S rRNA and 23S rRNA spacer region [223]. Positive and negative controls were included in each PCR cycle. Sequencing of positive amplicon’s was performed on both strands using primers homologous to those used for PCR on ABI 3730 capillary sequencers by Sequiserve, Vaterstetten, Germany. The PCR product purification protocol is documented under ‘Protocols- Protocol # 2’ on attached CD disk.

3.3.7 Pulsed Field Gel Electrophoresis (PFGE):
PFGE of DNA digested with the enzyme’s \(XbaI\) and \(BlmI\) was carried out in accordance with PulseNet protocol [38] using a CHEF-MAPPER system (Bio-Rad Laboratories, Hercules, CA). Isolate DNA was run on a 1% SeaKem gold agarose gel at 6.0 V/cm with an angle of 120° at 14°C for 20 hours. Initial switch time was 2.16 seconds with a final switch time of 54.17 seconds. Gels were stained with 10mg/ml of ethidium bromide and photographed. PFGE patterns were imported as TIFF files into Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and were interpreted accordingly. Dendrograms were constructed using the Dice similarity coefficient and the un-weighted pair group method with arithmetic averages (UPGMA). Related isolates were defined as having PFGE profiles with a >85% similarity [285].

3.3.8 Plasmid analysis:
Plasmid DNA was extracted from isolates using an Alkaline Lysis method as previously described [216]. Test DNA was run on a 0.7% gel for 5.5 hours at 70Volts. After electrophoresis, plasmid DNA was visualised by staining in
1 mg/ml ethidium bromide and the image was captured using the Bio-Rad Gel Doc system. A standard curve was constructed using control plasmid extracts of NCTC 50012 (72 Mda- C1 R64), NCTC 50001 (62 mDA- C2 R1), NCTC-50083 (126 Mda- C3 Rts1) and NCTC 50005 (26 Mda- C4 R6K). The standard curve was used to size test plasmids.

3.3.9 Conjugation experiments:
Conjugation was carried out using the method of Hasman et al. 2005 [125]. Recipient and donor Salmonella were grown overnight in 10 mL Brain Heart Infusion (BHI) medium at 37°C with shaking. A plasmid-free amoxicillin-susceptible nalidixic acid and rifampicin resistant S. Dublin isolate, was used as the recipient for mating experiments. From overnight cultures in BHI broth incubated aerobically at 37°C, 100 µL of each ESBL-positive isolate was transferred to 5 mL of fresh BHI broth and incubated at 37°C for 4 hours. Thereafter 500 µL of each ESBL isolate was mixed with 500 µL of the recipient and the entire volume was inoculated on a fresh Mueller Hinton agar plate containing 5% Sheep Blood. The blood agar plates were incubated aerobically for 5 h at 37°C. Transconjugants were recovered by pipetting 1 mL of fresh BHI broth on the blood agar plates. After gentle mixing, 10 µL, 50 µL and 100 µL was transferred to selective LB agar plates containing cefoxitin (32 mg/L) and nalidixic acid (50 mg/L) or ampicillin (32 mg/L) and nalidixic acid (50 mg/L). Transconjugant plates were incubated overnight aerobically at 37°C. Transconjugant colonies were sub cultured on selective plates and thereafter frozen at -80°C for further analysis.

3.4 Results:

Results are summarised in Table 3.2.

3.4.1 Antimicrobial susceptibility profiles:
Four of seven S. Kentucky exhibited antibiogram 1, multi drug resistance with particular resistance to ampicillin, choramphenicol, sulphonamides, tetracycline and ceftazidime with intermediate resistance observed to cefotaxime (ACSuTCazCtx). ESBL production in these isolates was confirmed by TZ/ TZL Etest. The remainder exhibited antibiogram 2, resistance to ampicillin,
ceftazidime, cefotaxime and cefoxitin (AmpCazCtxFox). The presence of \( \text{bla}_{\text{ampC}} \) was indicated in these cefoxitin resistant isolates and was confirmed by PCR.

### 3.4.2 Genes responsible for ESBL production:

*DNA sequencing data are documented in ‘Chapter 3-DNA sequencing’ on attached CD disk.*

Four of seven isolates (antibiogram 1) were shown to harbour \( \text{bla}_{\text{SHV-12}} \) with three isolates (antibiogram 2) harbouring \( \text{bla}_{\text{CMY-2}} \). All seven isolates were negative for the left and right junctions of the integrative element termed Salmonella genomic Island 1 (SGI-1), \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{CTX-M Groups 1, 2, 8, 9 & 25}} \).

### 3.4.3 Class 1 integron content:

*DNA sequencing data are documented in ‘Chapter 3-DNA sequencing’ on attached CD disk.*

The Class 1 Integron’s identified in each isolate was determined to be unrelated to the ESBL phenotype observed. Class 1 Integron’s (C1I’s) were not detected in isolate’s 08-1060 and 09-171. One isolate (08-1259) harboured C1I’s containing variable regions of 763bp (resistance gene cassette A-RGCA) and 638bp (resistance gene cassette B-RGCB). Three isolates harboured RGC-B alone (09-031, 060 & 061). RGCA containing the \( \text{aadB} \) gene confers resistance to kanamycin and gentamicin and RGCB containing the \( \text{sat} \) gene confers resistance to streptothricin.

### 3.4.4 PFGE of *Salmonella Kentucky* ESBL -isolates

In order to examine the clonal relationship between these isolates PFGE first using \( \text{XbaI} \) and later using \( \text{BlnI} \) was carried out (Figures 3.2 & 3.3). All isolates analysed by \( \text{XbaI} \) exhibited \( \geq 92\% \) identity however they were not identical (Figure 3.2).\( \text{BlnI} \) PFGE provided limited additional discrimination amongst the collection (Figure 3.3).
3.4.5 Plasmid analysis and transfer of ESBL/ AmpC resistance
All S. Kentucky isolates had identical plasmid profiles regardless of ESBL/ AmpC phenotype. Each harboured two plasmids of 4.9kb and 130kb (Figure 3.4). All isolates readily transfered the β-lactam resistance phenotype to the S. Dublin recipient. However, upon plasmid analysis of the transconjugant S. Dublin both 4.9kb and 130kb from S. Kentucky donors were co-transferred; therefore at the moment it is uncertain which plasmid is conferring the resistance.
**Table 3.2** Summary of results from analysis of β-lactamase producing *S. Kentucky*.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Isolate</th>
<th>Date Isolated</th>
<th>Specimen</th>
<th>Antibiogram</th>
<th>Xba/ PFGE</th>
<th>Bln/ PFGE</th>
<th>β-lactamase</th>
<th>Class1 Integron</th>
<th>Variable region sequence</th>
<th>SGI</th>
<th>Plasmids</th>
<th>Resistance Transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>08-1060</td>
<td>07/10/2008</td>
<td>Whole Chicken</td>
<td>ACazCtxFoxCpd</td>
<td>A</td>
<td>A</td>
<td>CMY-2</td>
<td>ND</td>
<td>-</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>F2</td>
<td>08-1259</td>
<td>25/11/2008</td>
<td>Chicken Neck Skin</td>
<td>ACSuTCazCtxCpd</td>
<td>B</td>
<td>B</td>
<td>SHV-12</td>
<td>763&amp;638</td>
<td>‘aadB’ &amp; ‘sat’**</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>F3</td>
<td>09-031</td>
<td>16/01/2009</td>
<td>Poultry Broiler Dust</td>
<td>ACSuTCazCtxCpd</td>
<td>D</td>
<td>C</td>
<td>SHV-12</td>
<td>638</td>
<td>‘sat’</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>F3</td>
<td>09-060</td>
<td>03/02/2009</td>
<td>Whole Chicken</td>
<td>ACSuTCazCtxCpd</td>
<td>C</td>
<td>D</td>
<td>SHV-12</td>
<td>638</td>
<td>‘sat’</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>F4</td>
<td>09-061</td>
<td>03/02/2009</td>
<td>Whole Chicken</td>
<td>ACSuTCazCtxCpd</td>
<td>C</td>
<td>D</td>
<td>SHV-12</td>
<td>638</td>
<td>‘sat’</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>F5</td>
<td>09-135</td>
<td>18/02/2009</td>
<td>Poultry Broiler Dust</td>
<td>ACazCtxFoxCpd</td>
<td>D</td>
<td>E</td>
<td>CMY-2</td>
<td>763</td>
<td>‘aadB’</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
<tr>
<td>Unknown</td>
<td>09-171</td>
<td>06/03/2009</td>
<td>Whole Chicken</td>
<td>ACazCtxFoxCpd</td>
<td>D</td>
<td>E</td>
<td>CMY-2</td>
<td>ND</td>
<td>-</td>
<td>-ve</td>
<td>4.9 &amp;130kb</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* The values shown indicate the presence of 763-bp and 638-bp variable regions, the 638-bp variable region only, or the 763-bp variable region only. ND, Class 1 integron not detected.

** *aadB* gene confers resistance to kanamycin and gentamicin and *sat* gene confers resistance to streptothricin
Figure 3.2  XbaI PFGE cluster analysis of β-lactamase producing S. Kentucky

Dice (Opt:0.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]

<table>
<thead>
<tr>
<th>PFGE-XbaI</th>
<th>PFGE</th>
<th>PFGE-XbaI</th>
</tr>
</thead>
<tbody>
<tr>
<td>s08-1060.1</td>
<td>Poultry</td>
<td>s08-1259</td>
</tr>
<tr>
<td>s09-0060</td>
<td>Poultry</td>
<td>s09-0061</td>
</tr>
<tr>
<td>09-0135</td>
<td>Poultry</td>
<td>09-0171</td>
</tr>
<tr>
<td>S09-0031</td>
<td>Poultry</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3  *Bln*I PFGE cluster analysis of β-lactamase producing *S.* Kentucky

Dice (Opt:1.50%) (Tol 1.5%-1.5%) (H>0.0% S>0.0%) [0.0%-100.0%]
Figure 3.4 Plasmid profile of CMY-2 and SHV-12 producing *S.* Kentucky isolated from poultry in Ireland

<table>
<thead>
<tr>
<th>L</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.</td>
<td>Super coiled DNA Ladder 2-16kb</td>
<td></td>
<td></td>
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<tr>
<td>1.</td>
<td>PB1 108kb</td>
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</tr>
<tr>
<td>2.</td>
<td>08-1060 CMY-2</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>3.</td>
<td>08-1259 SHV-12</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>4.</td>
<td>PB2 93kb</td>
<td></td>
<td></td>
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<tr>
<td>5.</td>
<td>09-031 SHV-12</td>
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<tr>
<td>6.</td>
<td>09-060 SHV-12</td>
<td></td>
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<td></td>
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<tr>
<td>7.</td>
<td>PB4 189kb</td>
<td></td>
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</tr>
<tr>
<td>8.</td>
<td>09-061 SHV-12</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>9.</td>
<td>09-135 CMY-2</td>
<td></td>
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<tr>
<td>10.</td>
<td>09-171 CMY-2</td>
<td></td>
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<tr>
<td>11.</td>
<td>PB5 39kb</td>
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</table>

L. Super coiled DNA Ladder 2-16kb
3.5 Discussion:
In this study we have found ESBL producing *S.* Kentucky in a variety of samples isolated from poultry farms in Ireland over a five month period. Resistance transfer was readily achieved in the laboratory highlighting the ease of spread of this resistance determinant. We do not have any data relating to the health of the birds from which the samples came or on the use of extended-spectrum cephalosporins on these farms.

Class 1 Integrons were not associated with a specific ESBL gene in our isolates; however two Class 1 integron’s conferring resistance to kanamycin, gentamicin and streptothricin were identified. Streptothricin acetyltransferase (*sat*) has previously been detected in Salmonella; however, is still thought to be quite rare [286].

The serovar *S.* Kentucky has been regarded as uncommon in humans; however, the serovar has been reported from clinical samples [287]. Collard and colleagues in 2007 highlighted the consequences of multi drug resistant *S.* Kentucky in humans [29]. The *S.* Kentucky in these clinical specimens was shown to harbour *bla*$_{\text{CTX-M-1}}$. In addition, this particular *S.* Kentucky clinical isolate was resistant to ciprofloxacin and co-trimoxazole. This report documented treatment failure to all the recommended antimicrobials for treatment of Salmonellosis. This highlights the potential mortality associated with emerging multi drug resistant serovars of Salmonella, even those previously thought to be unsuccessful human pathogens.

Upon review of the literature it is evident that food animals are increasingly associated with pathogens harbouring ESBL’s [168]. In the United States *S. enterica* isolates with reduced susceptibility to ceftriaxone were detected in cattle [169]. *S.* Kentucky resistant to the quinolones was reported in Ethiopia in 2006 from the carcasses of slaughtered pigs [171]. The problem has also been detected in Portugal with the observation of *bla*$_{\text{CTX-M-9}}$ in *S.* Virchow from laying hens and *S.* Enteritidis in Broilers with *bla*$_{\text{SHV-12}}$ isolated from *S.* Rissen from pigs [170]. A previous report of a similar phenomenon with *S.* Virchow from poultry products in France, Belgium, The Netherlands and Ireland reinforces the public health significance of my research [4, 152, 168]. In the UK the first report of an ESBL
from livestock was in 2006, with the ESBL responsible being \textit{bla}_{\text{CTX-M}}^{17/18} from an \textit{E. coli} isolated from calves [151]. Furthermore, a Spanish group has reported the presence of ESBL producing \textit{Klebsiella pneumoniae} in cooked chicken and ESBL producing \textit{E. coli} in salads [288]. Inter species transmission of plasmids harbouring \textit{bla}_{\text{CMY-2}} from an \textit{E.coli} strain to a \textit{S. Newport} within the same chicken is further cause for concern [276].

The transmission of bacteria containing antimicrobial resistance genes such as ESBLs is of concern to public health. A potential source for transmission of antimicrobial resistance genes, and bacteria harbouring same, to humans is through the food chain, example- retail chicken meat [289, 290]. A recent study by Overdevest and colleagues has highlighted that the predominant ESBL genes detected among retail chicken samples and among hospitalised patients from in the Netherlands were identical [291]. In addition, MLST highlighted that \textit{E. coli} isolated from retail chicken and from patient samples in the Netherlands clustered closely together [291]. This was a similar finding to Vincent and colleagues in Montréal, Canada [292]. Vincent and colleagues presented evidence that \textit{E. coli} isolated from retail chicken was indistinguishable from \textit{E.coli} isolated from patients with UTI's [292].

In the 1980s it was realised that the same standardisation seen in human medicine for AST was required for veterinary practice [4]. In 1998, the CLSI formed a subcommittee on Veterinary Antimicrobial Susceptibility testing (V-AST). The V-AST of the CLSI began with the task of developing standardised methods and interpretative criteria for AST testing of pathogens in veterinary medicine [198]. The first of these standards were published in 1999 [199]. This has been recently replaced by the CLSI/ NCCLS M31-A3-Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals [200, 201]. A recent paper by Schwarz and colleagues details the shortcomings of antimicrobial susceptibility testing of bacteria isolated from animals [293]. In addition, Schwarz and colleagues highlight the importance of adhering to the most current version of applicable standards [293].
There were 4 and 5 pulsed field profiles determined by XbaI and BlnI PFGE, respectively. It would be interesting to carry out MLST on the collection to establish if the strains belong to a successful clone of S. Kentucky. The importance of successful bacterial clones in the dissemination of antimicrobial resistance determinants amongst Gram negative bacteria was recently discussed in a publication by Woodford and colleagues [294].

To date there have been no reports or investigations on the source of the S. Kentucky in most countries from which human infection has been reported. The most likely vehicle of transmission of such pathogenic strains is through the food chain, with poultry being the main cause for concern and to the best of our knowledge, this is the first report of ESBL harbouring S. Kentucky isolated from poultry samples. It is therefore essential that firstly we continue to monitor antimicrobial susceptibility among human, food and food animal Salmonella isolates in Ireland and secondly to ensure that we have an efficient and vigilant surveillance system in place to deal with such resistance threats once they are identified in the food chain.

3.6 Conclusions:

These results and other publications elucidate the importance of monitoring food animals and food products for the presence of antimicrobial resistant pathogens and implementing appropriate control measures to protect public health.
Chapter 4

A comparison of S1 pulsed field gel electrophoresis (PFGE) and alkaline lysis for routine plasmid analysis
4.1 Abstract:
Plasmid analysis is useful for comparison of isolates for epidemiological purposes and in understanding transfer of antimicrobial resistance determinants. This section of my PhD compares alkaline lysis (AL) against S1-PFGE for plasmid analysis. S1-PFGE allows for more accurate size determination and greater reproducibility and is more convenient than alkaline lysis.

4.2 Introduction:
Plasmids are important extra chromosomal genetic elements. The ability of plasmids to transfer rapidly within and between bacterial species/genera is important in the dissemination of antimicrobial-resistance and virulence determinants, in particular among the Enterobacteriaceae [120]. Plasmids harbouring antimicrobial resistance determinants such as the extended-spectrum β-lactamase (ESBL) gene bla<sub>CTX-M-15</sub> have been implicated in a number of outbreaks over the past decade [120, 295]. In addition, plasmids are associated with virulence determinants. For example- IncF plasmids, frequently identified in Salmonella enterica, Shigella spp and E. coli often encode virulence determinants such as bacteriocins, siderophores, cytotoxins and adhesion factors [296]. Plasmid profiles may also serve as a useful adjunct to other typing methods in determining relationships between isolates from a confirmed or suspected outbreak.

Alkaline lysis (AL) is a traditional method for isolation of plasmid DNA. AL was introduced in 1979 by Birnboim and Doly [297]. The principle of the AL procedure involves separating plasmid DNA from chromosomal DNA by manipulating the pH of protocol reagents. Plasmid DNA is subsequently precipitated using phenol: chloroform: isopropyl alcohol. Linear electrophoresis is used to separate the plasmid extracts on an agarose gel. However, there are a number of limitations of the alkaline lysis method. Firstly, the plasmid conformation may be altered during the procedure which can impact on reproducibility because the mobility of nicked, linear and super coiled DNA conformations are different in an agarose gel [298]. Secondly, carry-over of fragmented chromosomal DNA can mask the identification of plasmids similar in
size to fragmented chromosomal DNA. Thirdly, the extraction and size determination of larger plasmids is not accurate [298].

S1-PFGE was first described for plasmid extraction and analysis by Barton and colleagues in 1995 [298]. An agarose plug with cells of the test organism is prepared. The cells undergo lysis within the agarose plug to release the DNA. The agarose plug is treated with S1 nuclease which converts plasmid DNA into linear molecules. S1 nuclease is unique in that it can identify a single stranded portion present in the conformation of all super coiled plasmids and cleave the plasmid DNA at this point [299]. S1 nuclease has a self-limiting action and therefore it makes few cleavages in chromosomal DNA [298]. The plug is placed in an agarose gel and subjected to electrophoresis in a PFGE chamber. Following separation the linearized plasmid DNA is observed as bands in a very faint genomic DNA background. This method has been successfully applied in the isolation and detection of large plasmids, however, the method is also efficient for the isolation and detection of smaller plasmids [83].

Although both methods have been in use for some years we have not been able to identify a report of a direct comparison of the methods in terms of ease of use, accuracy and reproducibility of size determination. The aim of this study was to compare the relative performance of these methods in these terms.

4.3 Materials and methods:

A detailed list of the materials, equipment and methods used for this work are documented under ‘Chapter 4 Materials, recipes and equipment’ on attached CD disk

4.3.1 Bacterial strains:

Five quality control (QC) laboratory strains harbouring plasmids of known molecular weight were analysed in triplicate by both methods. The strains were PB1 (108kb); PB2 (93kb); PB3 (189kb); PB5 (39kb); NCTC 50192 (7kb; 38kb; 66kb; 154kb). Plasmid free E. coli J53 was included in all analyses as a negative control.
4.3.2 Plasmid analysis by alkaline lysis methodology

Plasmid DNA was extracted from isolates using an Alkaline Lysis Method as previously described [216]. Test DNA was run on a 0.7% gel for 5.5 hours at 70Volts. After electrophoresis, plasmid DNA was visualised by staining in 1mg/ml ethidium bromide and the image was captured using the Bio-Rad Gel Doc system. A standard curve was constructed using control plasmid extracts of NCTC 50012 (72Mda- C1 R64), NCTC 50001 (62mDA- C2 R1), NCTC-50083 (126Mda- C3 Rts1) and NCTC 50005 (26Mda- C4 R6K). The standard curve was used to estimate the size of test plasmids. Variation in size estimates within runs and between runs was determined for the two methods (Figures 4.1 and 4.2). For within run variation, 2 lanes in one gel were analysed (Figures 4.1 and 4.2).

Detailed protocols relevant to this section are documented under ‘Protocols-Protocol # 6’ on attached CD disk.

4.3.3 Plasmid analysis by S1-Pulsed Field Gel Electrophoresis (PFGE) methodology:

For S1-PFGE agarose plugs of bacterial strains were prepared using the method of PulseNet [38]. A 2.5mm slice of each agarose plug was excised and digested with 1U of S1 nuclease enzyme (Promega, Madison, USA). The Pulse Marker™ HindIII digested Lambda DNA (Sigma-Aldrich, USA) was included as a size marker for S1-PFGE analyses. Each S1-PFGE gel was run on a 1% Seakem Gold agarose gel (Lonza, Rockland, ME, USA). The PFGE run conditions were optimised for the detection of large and small plasmids using the CHEF mapper system (Bio-Rad laboratories, Hercules, CA, USA). Each run consisted of 13 hours, at 6V/cm, with an angle of 120º, a consistent run temperature of 14ºC and a switch time of 1-13 seconds. Standard curves were generated in Microsoft Excel for estimation of large (> 38kb) and small plasmids (< 38kb) detected by S1-PFGE.

---

12 Pulse Marker™ HindIII (0.1kb-200kb) is a mixture of Lambda DNA HindIII fragments, Lambda DNA and Lambda concatamers embedded in a 1% low melting point agarose. For a convenient reference point, the concentration of the Lambda DNA monomer (48.5kb) is increased so it is the brightest band in the reference marker.
Detailed protocols relevant to this section are documented under ‘Protocols-Protocol # 7’ on attached CD disk.

4.4 Results:

Results are summarised in Table 4.1 and 4.2.

Plasmid size determination by S1-PFGE was more accurate and reproducible compared with the alkaline lysis method (Table 4.1 and 4.2). In each individual S1-PFGE run, the estimated size of plasmids harboured by NCTC 50192 corresponded largely with the known plasmid size (Table 4.1 and 4.2). In contrast; for AL, there was a large deviation between the estimated and known plasmid size both within and between runs particularly for plasmids >38kb (Table 4.1 and 4.2). However, AL was more reproducible than S1-PFGE for resolving the 7kb plasmid. Plasmids were more clearly defined and better separated using S1-PFGE (Figure 4.1).
Figure 4.1 Representative gel of isolated plasmid DNA by S1-PFGE protocol

L= Pulse Marker™ HindIII digested Lambda DNA (0.1kb-194kb), PB1 (108kb); PB2 (93kb); PB4 (189kb); PB5 (39kb); C=NCTC 50192 (7kb; 38kb; 66kb; 154kb). Plasmid free *E. coli* J53 was included in all analyses as a negative control
Figure 4.2 Representative gel of isolated plasmid DNA Alkaline Lysis protocols

SCL = Super coiled DNA ladder, 2-16kb, PB1 (108kb); PB2 (93kb); PB4 (189kb); PB5 (39kb); C=NCTC 50192 (7kb; 38kb; 66kb; 154kb). Plasmid free *E. coli* J53 was included in all analyses as a negative control.
Table 4.1: Within run variability of plasmids isolated from NCTC 50192 using Alkaline Lysis (AL) and S1- Pulsed Field Gel Electrophoresis (S1-PFGE)

<table>
<thead>
<tr>
<th>Expected plasmid size NCTC 50192</th>
<th>Alkaline lysis</th>
<th>Run 1 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
<th>Run 2 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
<th>Run 3 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>154kb</td>
<td></td>
<td>120/180</td>
<td>42.42</td>
<td>0.28</td>
<td>190/190</td>
<td>0</td>
<td>0</td>
<td>179/125</td>
<td>38.1</td>
<td>0.25</td>
</tr>
<tr>
<td>66kb</td>
<td></td>
<td>37/70</td>
<td>23.3</td>
<td>0.43</td>
<td>48/35</td>
<td>9.1</td>
<td>0.22</td>
<td>70/50</td>
<td>14.1</td>
<td>0.23</td>
</tr>
<tr>
<td>38kb</td>
<td></td>
<td>13/50</td>
<td>26.1</td>
<td>0.83</td>
<td>26/15</td>
<td>7.7</td>
<td>0.37</td>
<td>38/28</td>
<td>7.07</td>
<td>0.21</td>
</tr>
<tr>
<td>7kb</td>
<td></td>
<td>6/6.5</td>
<td>0.35</td>
<td>0.05</td>
<td>6.8/6.8</td>
<td>0</td>
<td>0</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expected plasmid size NCTC 50192</th>
<th>S1 PFGE</th>
<th>Run 1 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
<th>Run 2 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
<th>Run 3 Lane A/B (kb)</th>
<th>SD</th>
<th>COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>154kb</td>
<td></td>
<td>156/156</td>
<td>0</td>
<td>0</td>
<td>156/156</td>
<td>0</td>
<td>0</td>
<td>157/157</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>66kb</td>
<td></td>
<td>63/63</td>
<td>0</td>
<td>0</td>
<td>63/65</td>
<td>1.4</td>
<td>0.02</td>
<td>64/64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>38kb</td>
<td></td>
<td>37/37</td>
<td>0</td>
<td>0</td>
<td>37/37</td>
<td>0</td>
<td>0</td>
<td>34/34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7kb</td>
<td></td>
<td>7/7</td>
<td>0</td>
<td>0</td>
<td>7/7</td>
<td>0</td>
<td>0</td>
<td>7/7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

SD= Standard deviation, COV= Coefficient of variation
### Table 4.2
Between run variability of plasmids isolated from NCTC 50192 and PB1, PB2, PB4 and PB5 using Alkaline Lysis (AL) and S1- Pulsed Field Gel Electrophoresis (S1-PFGE)

**Alkaline lysis**

<table>
<thead>
<tr>
<th>Plasmid size</th>
<th>Run 1 mean (kb)</th>
<th>Run 2 mean (kb)</th>
<th>Run 3 mean (kb)</th>
<th>SD</th>
<th>COV</th>
<th>AVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 50192 154kb</td>
<td>150</td>
<td>190</td>
<td>152</td>
<td>22.5</td>
<td>0.13</td>
<td>164</td>
</tr>
<tr>
<td>NCTC 50192 66kb</td>
<td>53.5</td>
<td>41.5</td>
<td>60</td>
<td>9.3</td>
<td>0.18</td>
<td>52</td>
</tr>
<tr>
<td>NCTC 50192 38kb</td>
<td>31.5</td>
<td>20.5</td>
<td>33</td>
<td>6.8</td>
<td>0.24</td>
<td>28</td>
</tr>
<tr>
<td>NCTC 50192 7kb</td>
<td>6.25</td>
<td>6.8</td>
<td>5.5</td>
<td>0.65</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>PB1 (108kb)</td>
<td>120</td>
<td>140</td>
<td>130</td>
<td>10</td>
<td>0.07</td>
<td>130</td>
</tr>
<tr>
<td>PB2 (93kb)</td>
<td>84</td>
<td>100</td>
<td>92</td>
<td>8</td>
<td>0.08</td>
<td>92</td>
</tr>
<tr>
<td>PB4 (189kb)</td>
<td>180</td>
<td>155</td>
<td>170</td>
<td>12.5</td>
<td>0.07</td>
<td>168</td>
</tr>
<tr>
<td>PB5 (39kb)</td>
<td>26</td>
<td>45</td>
<td>40</td>
<td>9.8</td>
<td>0.26</td>
<td>37</td>
</tr>
</tbody>
</table>

**S1 PFGE**

<table>
<thead>
<tr>
<th>Plasmid size</th>
<th>Run 1 mean (kb)</th>
<th>Run 2 mean (kb)</th>
<th>Run 3 mean (kb)</th>
<th>SD</th>
<th>COV</th>
<th>AVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 50192 154kb</td>
<td>156</td>
<td>156</td>
<td>157</td>
<td>0.57</td>
<td>0.003</td>
<td>156</td>
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<tr>
<td>NCTC 50192 66kb</td>
<td>63</td>
<td>64</td>
<td>64</td>
<td>0.57</td>
<td>0.009</td>
<td>64</td>
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<td>NCTC 50192 38kb</td>
<td>37</td>
<td>37</td>
<td>34</td>
<td>1.7</td>
<td>0.04</td>
<td>36</td>
</tr>
<tr>
<td>NCTC 50192 7kb</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>PB1 (108kb)</td>
<td>129</td>
<td>137</td>
<td>126</td>
<td>5.6</td>
<td>0.04</td>
<td>131</td>
</tr>
<tr>
<td>PB2 (93kb)</td>
<td>103</td>
<td>110</td>
<td>96</td>
<td>7</td>
<td>0.6</td>
<td>103</td>
</tr>
<tr>
<td>PB4 (189kb)</td>
<td>182</td>
<td>192</td>
<td>178</td>
<td>7.2</td>
<td>0.03</td>
<td>184</td>
</tr>
<tr>
<td>PB5 (39kb)</td>
<td>31</td>
<td>38</td>
<td>38</td>
<td>4</td>
<td>0.1</td>
<td>36</td>
</tr>
</tbody>
</table>

SD= Standard deviation, COV= Coefficient of variation
4.5 Discussion:

S1 nuclease isolated from the mould *Aspergillus oxyzae* is an enzyme that attacks single stranded DNA and RNA [300]. The enzyme introduces nicks into single stranded regions of plasmid DNA\(^\text{13}\). This results in the supercoiling being relaxed and the plasmid DNA is converted to a linear form.

The S1 PFGE protocol was originally employed to extract and analyse large molecular weight plasmids, generally > 100 kb, termed mega-plasmids [298]. However, this work illustrates that the technique can be successfully applied in the extraction and analysis of plasmids as low a molecular weight as 7kb. The S1-PFGE technique for extraction and analysis of plasmids is being reported more frequently in the literature for the analysis of plasmids harbouring antimicrobial resistance determinants [301, 302]. S1-PFGE exhibits the potential to allow plasmid extraction and analysis to be standardised between laboratories across the globe, facilitating the more accurate size determination of epidemic plasmids responsible for the dissemination of antimicrobial resistance genes. The ability to use agarose plugs for both PFGE and plasmid analysis may result in a reduced cost to the laboratory and reduced time in plasmid analysis. The plasmid extract on alkaline lysis is generally sufficient for one run only and therefore if the plasmid gel needs to be repeated another fresh extraction is required, whereas with S1-PFGE- the agarose plug is prepared and stored and there is sufficient quantity for two S1-PFGE and two standard PFGE runs per isolate agarose plug.

There are a number of aspects of the S1-PFGE technique that culminate in greater reproducibility and accuracy compared to the alkaline lysis technique for the analysis of plasmids. These aspects include:

- The method of plasmid extraction used in S1-PFGE: during this step, all bacterial cells are immobilized in an agarose plug. Once the cells are lysed all cellular debris and
- The enzyme used in S1 PFGE: S1-Nuclease converts supercoiled DNA to linear DNA. Therefore S1-PFGE is more efficient and accurate in determining the size of a plasmid. A difficulty with alkaline lysis is that

\(^{13}\)Plasmids possess a single-strand initiation site (ssi) for the priming of DNA replication. The ssi has been located near the ori in plasmids.
different conformations of the same plasmid can result in difficulty in interpreting results and with reproducibility of the test.

- The apparatus used in S1-PFGE: S1-PFGE using the CHEF Mapper® apparatus enables separation of large and small DNA fragments with better resolution, speed, and accuracy than traditional electrophoresis methods. S1-PFGE in particular is superior for the analysis of large plasmids due to the incorporation of the CHEF Mapper® apparatus.

- The method of determining plasmid size in S1-PFGE: Standard curves were generated in Microsoft Excel for estimation of large (≥ 38kb) and small plasmids (< 38kb) detected by S1-PFGE. This method of size determination is more accurate than that employed for alkaline lysis. With alkaline lysis, 4 cycle semi-log graph paper is used to manually generate a standard curve with estimation of plasmid sizes generated from same. This is subject to a certain level of human error.

- Effect of chromosomal DNA in S1-PFGE interpretation: The carry-over of fragmented chromosomal DNA in alkaline lysis can mask the identification of plasmids similar in size to fragmented chromosomal DNA.

4.6 Conclusions:

In conclusion, S1-PFGE is more accurate and reproducible for estimation of plasmid size across a broad size range and provides greater image clarity. Based on the experience of PulseNet it is likely that the S1-PFGE may be readily standardised to achieve inter-laboratory agreement which is essential for application of plasmid profiles in investigation of large outbreaks. We suggest that S1-PFGE should be accepted as the preferred method in laboratories using plasmid profiles for public health epidemiology.
Discussion
Recap on the goals of my research:

There were three main findings of my PhD research. I have provided evidence that with respect to cefepime as substrate OXA-1 mediated resistance is significantly inhibited by clavulanic acid. Prior to my research OXA-1 was not reported to be significantly inhibited by clavulanic acid. This work represents the first report in the literature of ESBL and plasmid-mediated ampC in *Salmonella* Kentucky isolated from poultry. This was a significant finding as it highlighted the concern that the scientific community has regarding poultry as a reservoir of antimicrobial resistant *Salmonella*. In addition this finding elucidated concerns regarding the role that poultry could play in dissemination of antimicrobial resistant *Salmonella*; and in fact *Enterobacteriaceae* in general; into the food chain. Finally, my research validated an alternative method for plasmid extraction and analysis in our Laboratory. Previous to this work there was no published head to head comparison of alkaline lysis and S1-PFGE as plasmid extraction/analysis tools.

β-lactamase mediated resistance in *Salmonella enterica*:

Non-typhoid Salmonellosis (NTS) is generally uncomplicated and self-limiting. There is generally no clinical benefit of antimicrobial therapy. Antimicrobials such as the third generation cephalosporins, trimethoprim-sulfamethoxazole or ciprofloxacin are prescribed in invasive case of NTS and therefore antimicrobial resistance is of clinical consequence in this situation [303].

There is a vast reservoir of non-typhoid *Salmonella* in animals that poses risks for dissemination to humans. In Europe the majority of NTS cases in humans are attributed to zoonotic transmission [304]. *Salmonella* Enteritidis and *Salmonella* Typhimurium are the serovars most often associated with animals intended for food consumption that are linked with human illness. Domestic pets are also a source of salmonellosis in their human owners [305-308].

Resistance to β-lactam antimicrobial agents in a collection of *S. Typhimurium* isolates represents the major part of my work. The majority of the collection of *Salmonella* Typhimurium was from sub-Saharan African and is of the MLST
type ST313. The one Irish isolate in the collection was identified as ST19 which is one of the most common ST’s in the *Salmonella enterica* MLST database. ST19 is the predecessor of ST213, ST302 and ST429 and is not closely related to ST313. ST313 is a strain of *S. Typhimurium* which has rarely been identified outside of Sub Saharan Africa [269]. *Salmonella* Typhimurium ST313 is associated with bacteraemia, and/ or meningitis and with septic arthritis also reported. Gastroenteritis presents in only half of the reported cases. It is suggested that infection with MDR Typhimurium ST313 may result in more invasive disease with its predominant transmission from person to person attributed to rapid adaptation to its human hosts [269]. *S. Typhimurium* ST313 differs from strains of *S. Typhimurium* (such as ST19) causing classical gastroenteritis due to the presence of a novel selection of prophage elements coupled with selective genomic degradation; both yielding a successful clonal group that may continue to evolve in the way that *Salmonella* Typhi, Paratyphi A and Gallinarum have in order to adapt to their specific ecological niche [269]. Antimicrobial resistance in this clonal group may therefore be a particular cause for concern.

Poppe and colleagues recently reported that *S. Newport* can become resistant to extended-spectrum cephalosporins and other antimicrobials by acquiring a conjugative drug resistance plasmid from *E. coli* in a turkey poult intestinal tract [276]. Antimicrobial resistance in *Salmonella* has been reported since the 1960s [309]. The occurrence and frequency of antimicrobial resistant *Salmonella* and the types of antimicrobial resistance profiles vary between countries and continents. Globally the most common serotypes associated with antimicrobial resistance and that are frequently isolated from animals intended for human consumption are *S. Typhimurium*, *S. Enteritidis*, *S. Heidelberg*, *S. Hadar*, *S. Virchow*, *S. Newport* and *S. Welteverden* [4]. Studies carried out in the United Kingdom highlighted that the level of antimicrobial resistant *Salmonella* increased by over 50% from 1981 compared to 1988 [4]. There is a clear relationship between the presence of antimicrobial resistance in *Salmonella*, the severity of disease and the outcome associated with such an infection. The CDC for example reported a higher fatality rate in Salmonellosis cases (1971-1983)
where there was associated antimicrobial resistance when compared to fully susceptible infections, 4.2% versus 0.2% respectively [310]. Further analysis of Salmonellosis cases during this period from the community and hospital setting highlighted that 57% of cases infected with antimicrobial resistant Salmonella required hospitalisation as opposed to only 24.5% requiring hospitalisation when infected with susceptible strains. This trend was confirmed in a more recent review (1984-2002) of 24 Salmonella outbreaks carried out by the CDC [310, 311]. In addition a study carried out by Helms and colleagues in Denmark during 1995-1999 analysed the mortality rates associated with antimicrobial resistant Salmonella [312, 313]. The results of this work highlighted that patients infected with pan susceptible S. Typhimurium were 2.3 times more likely to die within 2 years (compared to control group) whereas patients infected with S. Typhimurium DT104 (ACSSuT) were 4.8 times more likely to die [313].

Extended-spectrum cephalosporins are an important option for treatment of invasive salmonellosis. They are widely available, costs have declined in recent years, they are comparatively safe to use in adults and children and as they have a broad spectrum of activity so that they are useful for empiric therapy when a specific infecting pathogen has not yet been detected. Acquired resistance to a member of this class of antimicrobial agents is therefore of concern. β-lactamase mediated resistance has become prevalent in Salmonella serovars since the first identification of SHV-2 in S. Wien in the 1980s [148]. In 2004, forty three countries had reported having a public health problem with extended-spectrum cephalosporin resistance in Salmonella [149]. Unlike many Enterobacteriaceae, Salmonella do not possess an intrinsic β-lactamase, therefore all β-lactamase mediated resistance in Salmonella has been acquired through the dissemination of β-lactamase genes into the genus.

The role that plasmids and mobile genetic elements play in this situation is apparent in the work presented here. In all of the ST313 S.Typhimurium isolated from Sub Saharan Africa over a 10 year period and a distinct ST19 S.Typhimurium isolated from a patient in Ireland- a common plasmid was associated with the ‘cefepimase’ phenotype (pFEP39). Using PCR based
replicon typing; the pFEP39 was identified as a novel IncH12/W plasmid. Multi-replicon plasmids have been reported in *Salmonella* previously including Inc HI2 plasmids carrying ESBL genes [120, 267]. IncW plasmids exhibit a broad host range including *S. Typhimurium* [268]. However, this is the first report of a multi replicon plasmid with the Inc HI2 /Inc W replicon combination. Multi-replicon plasmids offer a significant advantage over single replicon plasmids in terms of an expanded host range. The role that mobile genetic elements such as plasmids play in the dissemination of β-lactamase mediated resistance determinants is also apparent in the study of β-lactamase producing *S. Kentucky*. All isolates of *S. Kentucky* were isolated from different geographical locations in Ireland from different time periods and harbored similar sized plasmids. ESBL *bla*<sub>SHV-12</sub> and AmpC *bla<sub>CMY-2</sub>* genes were shown to be located on transferable plasmids harboured by *S. Kentucky*.

Routine testing for ESBL in *Enterobacteriaceae* is no longer considered essential for clinical purposes by either CLSI or EUCAST (refer to Chapter 1 section 1.7.2) now that interpretive breakpoints for cephalosporins have been substantially lowered. However, ESBL confirmation remains highly recommended for epidemiology and infection control purposes (refer to Chapter 1 section 1.7). The presence of different types of β-lactamase enzymes in Salmonella provides challenges in characterising these antimicrobial resistance determinants in the laboratory. For example, confusion can occur when bacteria possess both an ESBL gene and a plasmid-mediated AmpC. In these instances inhibitory tests using a third generation cephalosporin and an inhibitory substance such as clavulanic acid are inconclusive (refer to Chapter 1 section 1.7.2.2.1)

- **CMY β-lactamase (refer to Chapter 1 section 1.3.5)**

Plasmid-mediated AmpC’s are most often found in nosocomial and non-nosocomial *Enterobacteriaceae* isolates that do not possess a chromosomal *ampC* or, as in the case of *E. coli*, which have a non-inducible *ampC*. The plasmid-mediated AmpC CMY-2 is a frequently encountered cause of β-lactam resistance in non-typhoidal *Salmonella* in many countries across the globe [314].
The \( \text{bla}_{\text{CMY-2}} \) gene is the most widely disseminated \( \beta \)-lactamase in Salmonella [149]. The CMY group comprise the majority of the plasmid-mediated AmpC’s with 64 variants identified to date [66]. In the USA the most common plasmid-mediated AmpC’s are CMY-2, ACT-1, DHA-1 and FOX-5, in the United Kingdom CIT-like, CMY-2, CMY-7, CMY-21, CMY-23, ACC-like, FOX-like and DHA-like and in Canada CMY-2 predominates [97]. The plasmid replicons associated with CMY-2 dissemination is interesting. A recent study by Martin and colleagues demonstrated that CMY-2 dissemination among \( E. \ coli \) and Salmonella isolated from food and food products in Canada was associated with 5 different replicon types [280]. Martin and colleagues have emphasized the potential challenges that a diverse range of plasmids pose for containment of antimicrobial resistance determinants such as CMY-2. Prior to this work; however, plasmid-mediated ampC had not been reported in Salmonella from poultry. The work on \( S. \) Kentucky presented here provides evidence that CMY-2 was successfully disseminated in poultry farms across Ireland between 2008 and 2009. It is a curious finding the phenomenon then disappeared (personal communication NSRL).

- **SHV \( \beta \)-lactamase (refer to Chapter 1 section 1.3.3)**

The SHV-1 \( \beta \)-lactamase was originally identified on the chromosome of \( Klebsiella \) species. Since this, the SHV gene has mobilised from the chromosome of \( Klebsiella \) and disseminated amongst other members of the \( \text{Enterobacteriaceae} \). The dissemination of SHV from \( Klebsiella \) species and other members of the \( \text{Enterobacteriaceae} \) into Salmonella by mobile genetic elements have serious consequences for public health. The first report of SHV in Salmonella in Ireland was associated with foreign travel [157]. The SHV-12 gene identified in this study was harbour by \( S. \) Worthington isolated from a patient with associated foreign travel to India [157]. In addition, by documenting this phenomenon in isolates from poultry it expands the range of species demonstrated to harbour \( S. \) Kentucky producing SHV-type \( \beta \)-lactamase.
OXA β-lactamase- (refer to Chapter 1 Sections 1.3.5 and 1.3.5.1)

Epidemiological data on the geographical spread of OXA β-lactamases is lacking, perhaps due to the difficulty in their identification in the clinical laboratory. Many of the OXA β-lactamases have been reported on one or two isolated occasions. Many of the more recently identified OXA β-lactamases have originated in France and Turkey [76, 77]. The number and clinical significance of OXA β-lactamases is increasing, especially as their host repertoire broadens from *Pseudomonas* spp. and *Acinetobacter* spp to other members of the *Enterobacteriaceae*.

The OXA-1 enzyme has several notable differences compared to the other Class D β-lactamases which have been assessed. The OXA-1 enzyme is a monomer. The OXA-1 Ω loop possesses six additional residues compared to OXA-10 (therefore OXA-1 exhibits a longer Ω loop, however the Ω loop is still shorter than in Class A enzymes). OXA-1 possesses an aspartate residue at position 66 (D<sup>66</sup>), which is buried in the floor of the OXA-1 active site. These residues are known to play an important role in substrate specificity. Leonard and colleagues hypothesize that interactions between D<sup>66</sup> and adjacent amino acids are required for OXA-1 mediated resistance to the fourth generation cephalosporin cefepime [71].

The Arg244 (R<sup>244</sup>) residue is a conserved residue in the B4 β strand of Class A β-lactamases, but is absent in the structure of OXA-1 and in Class C β-lactamases [315]. R<sup>244</sup> can interact with the C3 carboxylic acid group of β-lactams. This residue is believed to be responsible for forming critical hydrogen bonds with the C-3 carboxylate of clavulanic acid. However, there have been intense speculations as to the exact role of R<sup>244</sup>, with some groups disregarding its direct involvement in β-lactam hydrolysis and turnover [316, 317]. It has been suggested that mutagenesis studies to determine which exact residues are essential for clavulanic acid binding and hence inhibition of the β-lactamase would not be easily achieved as the residue is most likely critical for enzyme integrity and therefore mutant enzymes cannot be readily selected [316]. There
have been no such studies to this effect to investigate the function of residues in the Ω loop of OXA-1 enzyme in binding with the clavulanic acid molecule.

The OXA-1 Ω loop has the following residues D-K-E-R-N-N. Kinetic analysis illustrates that these residues do not allow OXA-1 to turnover extended-spectrum cephalosporins with large C6/ (7) side chains [101]. Cefepime differs from these cephalosporins as it has a less bulky chain at the C7 position, which enables more efficient penetration of the Gram negative cell. OXA-1 is capable of more efficiently hydrolysing cefepime as opposed to third generation cephalosporins probably due to the less bulky side chain at position C7 of the cefepime cephalosporin nucleus. The introduction of an oxyimino group into the side chain at position 7 of the cephalosporin nucleus or at position 3 of the monobactam nucleus is the major means of protecting a β-lactam bond from hydrolysis by the serine β-lactamases.

Prior to this work there were no published reports stating that OXA-1 was significantly inhibited by clavulanic acid. This initially caused confusion as the data clearly suggested that the phenotype observed was one of an ESBL; one that OXA-1 is not known to confer. Based on the data presented here and published, it is suggested that depending on the substrate hydrolysed, OXA-1 is capable of significant inhibition by clavulanic acid.

A recent study by Beceiro and colleagues reported a similar phenotype in E.coli isolated from a wound of a patient in Waterford, Ireland and the urine of a patient from Scotland [318]. Both isolates exhibited non-susceptibility to cefepime and cefpirome (4 and 8 mg/L) with significant inhibition in the presence of clavulanic acid (32 fold reduction in cefepime MIC in presence of clavulanic acid). Both strains were susceptible to ceftazidime and cefotaxime. Both strains harboured OXA-1 and TEM-1. The mechanism of ‘cefepimase’ in these isolates was attributed to two different mechanisms. One was attributed to (Scottish isolate) overexpression of TEM-1 due to a strong P5 promoter. The other (Irish isolate) was attributable to overexpression of OXA-1 coupled with reduced expression of
The mechanism of over expression of OXA-1 in this study was not further examined.

From a diagnostic point of view the use of cefepime +/- clavulanic acid is essential is detecting this phenotype in the clinical laboratory. However, this is not often included in the repertoire of ESBL detection and confirmation techniques by most laboratories therefore it is difficult and to accumulate data on the epidemiology of such resistance determinants.

- **Molecular typing of ‘cefepimase’ producing S. Typhimurium**

In this study of 19 isolates of S. Typhimurium, XbaI PFGE was most discriminatory (14 types) followed by MLVA (13 types) and MLST (2 types) Foley and colleagues applied PFGE, Rep-PCR and a form of sequenced based typing based on MLST to determine which typing technique offered the greater level of discrimination when applied to a collection of S. Typhimurium [319]. They report the ‘MLST’ like method as most discriminatory; however, this finding is not comparable with the MLST method used in this project or in most published papers because they used non-house keeping gene sequences [319].

Similar to our finding Sukhnandan and colleagues reported that MLST (using housekeeping genes only) exhibits limited ability to discriminate within Salmonella serotypes when compared to sub typing methods such as PFGE [320]. MLST offers significant advantages over PFGE when studying the phylogeny of bacteria; however, many scientists incorrectly continue to use PFGE data for the construction of phylogenetic trees or evolutionary analysis when the technique is clearly limited in this application [321].

In relation to XbaI PFGE, the digested genomic fragments in the region of c. 200kb - c. 1000kb were similar among all ‘cefepimase’ S. Typhimurium with the major differences in Pulse field Profiles attributed to the smaller molecular weight fragments (< c. 200kb). This trend was also observed with BlnI PFGE with a similarity in the fragment sizes between c. 400kb and c.1000kb and a diversity noticed in the smaller fragments c. < 240kb. This observation is similar
to that found by Kariuki and colleagues in a study of the genetic diversity of \textit{S. Typhimurium} in Kenya over a ten year period [218]. An explanation for the diversity seen in the lower molecular weight fragments may be due to the presence of plasmids or prophage elements.

Cooke and colleagues highlighted that MLST compared to PFGE and MLVA is less discriminatory when applied to \textit{S. Typhimurium DT104} isolates [322]. Cooke identified that DT104 \textit{S. Typhimurium} in their study were all ST19, however all had distinct PFGE patterns, again with the majority of variation occurring in the mid- lower molecular weight fragments. Cooke found that certain DNA signatures (prophage elements) in \textit{S. Typhimurium DT104} are potentially subject to DAM methylation; therefore protected from \textit{XbaI} digestion. For example the presence of prophage 3 generates a 439kb band that is present on PFGE gels post \textit{XbaI} digestion of genomic DNA. This was similar in a large collection of DT104 isolates [322]. The group also investigated the presence of the prophage elements in a DAM negative mutant LT2 \textit{S. Typhimurium}, illustrating that this particular strain did not exhibit the banding pattern of the Dam positive DT104 \textit{S. Typhimurium} (i.e. 439kb band representing prophage 3) [322]. The experiment was repeated with DT10 and DT11 \textit{S. Typhimurium} and the 439kb prophage 3 element was absent, however a 389kb element was detected, thought to be prophage 5 [322]. The presence of large plasmids was also highlighted as a cause for PFGE variation in Cooke’s investigation [322]. Cooke hypothesized that this could be a cause for PFGE pattern variation in other strains of \textit{S. Typhimurium}.

- **Factors that can drive β-lactamase resistance**

Antimicrobial resistant bacteria are spread by a complex interaction between selective pressure\textsuperscript{14}, successful dissemination of bacterial clonal groups and or epidemic plasmids. Examples include the internationally disseminated \textit{E. coli} clone 025:H4-ST131 harbouring \textit{bla}_{CTX-M-15}. [323]. Initially, the process commences with the introduction of a resistant organism into a population-

\textsuperscript{14} Selective pressure relates to the environmental conditions that permit an organism to survive and proliferate. Antimicrobial use is an example of selective pressure. The populations of susceptible organisms are killed off, allowing the resistant population to predominate.
whether this be in the health care setting or in the animal population. Thereafter, selective pressure such as antimicrobial use in a healthcare setting/ veterinary sector will permit proliferation of a resistant organism/plasmid. From there the next step is clonal dissemination. This can occur due to inadequate hygiene practices, infection control inadequacies and/or contaminated equipment [323, 324]. Antimicrobial stewardship is key in addressing the issue of antimicrobial selective pressure.

- The gaps in our knowledge of β-lactamase mediated resistance

The role that antimicrobial prescribing practices have on the selection and dissemination of ESBL resistance determinants within the food chain in Ireland requires further analysis. Many of the cephalosporin agents licensed for use in Ireland in veterinary medicine have only been in use since c. 2007. The effect that their use has in selection and dissemination of antibacterial resistance determinants within the farm environment, local environment and through the food chain has not been assessed. In the USA a recent study by Singer and colleagues found that *E. coli* isolated from the faeces of untreated cows were susceptible to ceftiofur, with *E. coli* isolated post treatment with ceftiofur being resistant [325]. The authors did admit that the level of the study was not sufficient to categorically state there was a causal link between antibacterial uses and the emergence or amplification of antibacterial resistance.

Retrieval of exact data on the level of antimicrobial consumption in the veterinary sector is difficult at the moment and the reported statistics in the limited published work in this area needs to be interpreted with caution. In 2009 the European commission gave the European Medicines Agency (EMEA) a mandate to take the role of collecting data on the sales and use of antimicrobials in veterinary medicine. The EMEA has set up the Committee for Medicinal Products for Veterinary Use (CVMP) and in 2009 coordinated an advisory group- Scientific Advisory Group on Antimicrobials (SAGAM), which aided in analysis of the use of cephalosporin’s in veterinary medicine [326]. The EMEA implemented a coordinated data collection effort in 2011 with planned reports due by the end of 2012 [327]. This data will be essential to allow an accurate
assessment of the occurrence of antimicrobial resistance in zoonotic and animal disease agents and the potential link with antimicrobial consumption.

- Global approaches to antimicrobial resistance and the challenges of antimicrobial development

The European Surveillance of Antimicrobial Consumption (ESAC) and the World Health Organization (WHO) Third Global Patient Safety Challenge run programmes that are vital in monitoring antimicrobial prescribing practices. Both the ESAC and WHO play their part in protecting currently approved antimicrobials from becoming obsolete.

The issue of growing levels of antimicrobial resistance worldwide in bacterial pathogens is further compounded by the lack of new antimicrobials available on the market [328]. There are a number of antimicrobials in development to date in the line of treating Gram positive infections. However, there is minimal progress in relation of development of drugs for treatment of Gram negative infections. Development of antibacterial agents targeted towards multi drug resistant Gram negative’s (MDRGN’s) has reached an all-time low, with no agent with a novel mechanism of action against MDRGN’s being filed for registration for over 30 years [59].

Some of these agents in development and directed towards MDRGN’s are analogues of existing molecules and therefore are not exactly novel approaches; for example- ceftazidime in combination with a β-lactamase inhibitor NXL-104. The β-lactam/ β-lactamase inhibitor approach has not resulted in a new product since 1993 when piperacillin/ tazobactam were approved for clinical use [329]. Other examples of agents that are in the research and development stages are- a lipopeptide agent (being developed by Cubist), a non β-lactam penicillin-binding protein inhibitor (being developed by Novexel) and a membrane biosynthesis inhibitor (being developed by Achaogen). All exhibit promising activity towards the Enterobacteriaceae [329]. Another interesting approach is that suggested by
David Livermore in his recent publication-‘Temocillin Revived’ [59]. In this work the author discusses that to meet the current demand in treating MDRGN’s we should re-examine older, ‘forgotten’ compounds that may now be of benefit. One such compound is Temocillin.

There are, however, measures being taken to address the issue of antimicrobial resistance. In late 2009 a summit was held between the United States and the European Union to establish a transatlantic task force to tackle antimicrobial resistance [330]. The ‘immediate goal’ of this task force is ‘the development of ten novel drugs by 2020’. This may prove difficult.

- The limitations of my research

There are a number of areas that were not pursued because of time constraints. Firstly, in relation to the work on the ‘cefepimase’ phenotype. My sample set was from Sub Saharan Africa and Ireland. This collection was selected while we were using the PM/PML Etest in our confirmation of ESBL detection. It would have been valuable to collaborate with those that hold collections of relevant isolates in other countries. It would have been of value to examine the outer membrane proteins (OMP) of the isolates to determine if changes in OMPs (whether expressed or not) may have explained the varying resistance to cefepime amongst isolates in the collection (8-32μg/ml) (refer to Chapter 2 Table 2.8). In addition, it would have been valuable to investigate the MIC of each transconjugant harbouring pFEP39 towards a panel of antimicrobial agents, for example the agents used to assess the antibiogram in donor isolates (refer to Chapter 2 Section 2.3.2). This experiment would illustrate if pFEP39 conferred resistance to other classes of antimicrobials in addition to the β-lactams. Time constraints prevented this work from being carried out. Kinetic studies of enzyme –substrate and enzyme-substrate-inhibitor interactions would have helped to clarify the interaction between cefepime, clavulanic acid and OXA-1. Although a sequence associated with strong promoter activity was identified upstream of blaOXA-1, there was not sufficient time to study gene expression or levels of enzyme production. CMY-2 was detected in Salmonella Kentucky as part of Chapter 2’s investigation, however, there was insufficient time to investigate and
determine other plasmid-mediated AmpC’s present in Salmonella identified as phenotypic ampC producers from the NSRL bank. In relation to the validation of S1-PFGE as a method of plasmid extraction and analysis, a larger sample size would have been more optimal to prove the accuracy of the technique for sizing plasmids.

- **Future work in this area**

If I had more time on this project there are a number of experiment’s I would like to complete:

- It would have been valuable to investigate the MIC of each transconjugant harbouring pFEP39 towards a panel of antimicrobial agents, for example the agents used to assess the antibiogram in donor isolates, however time contrainsts preventing this being carried out.

- It would be interesting to carry out kinetic studies of the OXA-1 enzyme. OXA-1 enzyme is not known to be inhibited by clavulanic acid. The result of my thesis research has demonstrated that when cefepime is the substrate, OXA-1 (in the genetic context identified in this thesis) is significantly inhibited by clavulanic acid.

- As the cefepime MIC of the individual *S. Typhimurium* in the collection studied varied, it would be interesting to carry out RT-PCR- to compare the expression of OXA-1 amongst my collection.

- It would be interesting to investigate the effect that outer membrane porin (OMP) loss has on the level of cefepime resistance amongst the collection of *S. Typhimurium.*
In conclusion:

The research that I have executed as part of this PhD thesis has contributed to the field of ESBL detection in Salmonella by:

1. My research identified an ESBL/ AmpC not previously reported in Salmonella from food animals in Ireland. This was the first report of plasmid-mediated cephalosporin resistance in *S. Kentucky* from poultry. Dissemination of such acquired resistance is a threat to both animal and human health and should elicit a vigorous response at national and international level. This finding further highlights the importance of the Reference Laboratory in monitoring isolates and alerting relevant authorities. Interestingly, the emergence and dissemination of the ESBL/ AmpC phenotype in *S. Kentucky* in Ireland has not since been identified. This finding was published in the Journal of Antimicrobial Agents and Chemotherapy – Reference Appendix # 1.

2. My research highlighted that a commonly encountered β-lactamase *bla*<sub>OXA-1</sub>, has the potential in the appropriate context to be associated with an unusual ESBL phenotype that is easily missed by standard methods for ESBL detection. It is uncertain how important this finding is for therapeutics in the context of recent updates on susceptibility test methods; however, it raises questions regarding the accepted view of OXA-1 as not being inhibited by clavulanic acid and the importance of this finding for the routine identification and confirmation of ESBL producers in the clinical laboratory and the research laboratory. This finding was published in the Journal of Diagnostic Microbiology and Infectious Disease – Reference Appendix # 2.

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3. My research validated S1 PFGE for the extraction and analysis of plasmids as more reproducible and accurate than alkaline lysis. This finding is submitted for publication in the BioMed Central Journal entitled ‘Research Notes’.
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First Report of Extended-Spectrum-β-Lactamase-Producing Salmonella enterica Serovar Kentucky Isolated from Poultry in Ireland

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Therapy of invasive human salmonellosis is complicated by increasing antimicrobial resistance. Food animals are the principal source of infection with nontyphoidal Salmonella. We report the emergence of broad-spectrum-cephalosporin resistance in Salmonella enterica serovar Kentucky in poultry in Ireland.

Increasing antimicrobial resistance among isolates of Salmonella from food animals is a significant public health concern because of the potential to cause human infection. Broad-spectrum cephalosporins, together with fluoroquinolones, were until recently considered reliable agents for empirical therapy of invasive salmonellosis. Cephalosporin-resistant Salmonella enterica bacteria that disseminate in food animals therefore have the potential to cause human infection, for example, cephalosporin-resistant Salmonella enterica serovar Virchow (poultry in France) and Salmonella enterica serovar Newport (cattle in the United States) (1, 3, 5, 12).

Prior to 2008, resistance to cephalosporins was not reported for isolates of Salmonella from food animals in Ireland. Cephalosporin resistance has been detected in isolates from humans associated with travel outside Ireland. From January 2000 to September 2008, we analyzed 925 isolates of Salmonella enterica serovar Kentucky of human (n = 60) and animal (n = 865) origin, with all isolates being susceptible to cephalosporins. Between October 2008 and March 2009, 7 of 115 S. Kentucky isolates were resistant to cefotaxime and ceftriaxone. Cephalosporin-resistant isolates were from chicken neck skin, whole birds, and描写者.

A number of human cases of S. Kentucky infection have occurred during most years in Ireland, and human infection has also been documented to occur elsewhere (5, 7). Collard and colleagues in 2007 (9) reported on blstTCM, containing S. Kentucky bacteria that were cointant with ampicillin and cefotaxime. Other studies have discussed the importance of emerging serovars of Salmonella to human health (7, 9, 17, 21).

Identification and serotyping of isolates were performed by standard methods. Testing of susceptibility to 14 antimicrobial agents was performed by Clinical and Laboratory Standards Institute (CLSI) disk diffusion methods (8). Cephalosporin-resistant isolates were assessed for extended-spectrum-β-lactamase (ESBL) production by using cefepoxide (30 μg) and by using cefpodoxime plus clavulanic acid (10 μg/1 μg) and ESBL. Etests with cefazidime, cefotaxime, and cefepime in each case with and without clavulanic acid (AB Biodisk, Solna, Sweden). AmpC production was suspected in the absence of clavulanic acid potentiation of cephapirin and additional resistance to cefotaxime for the 12-field gel electrophoresis (PGFE) was performed by the PulseNet method with XbaI and BlnI, and the results were analyzed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium). Plasmids were prepared as previously described (20) and visualized on 0.7% Tris-acetate-EDTA gels by staining with 1 mg/ml ethidium bromide. Size was estimated using a standard curve constructed from plasmids from strains NCTC 50121 (72 MDa), NCTC 5000 (62 MDa), NCTC 50583 (126 MDa), and NCTC 50005 (26 MDa). Conjugation was carried out using the method of Hasman et al. (13). A plasmid-free, amoxicillin-susceptible, and nalidixic acid- and rifampin-resistant Salmonella enterica serovar Dublin isolate was the recipient for mating experiments. Transconjugants were selected on LB agar plates containing cefotaxime (32 μg/ml) and nalidixic acid (50 μg/ml). Genomic DNA was extracted with a QIAamp DNA minikit (Qiagen, Inc., Valencia, CA). Absence of inhibitors of PCR was confirmed by amplification of 16S rRNA and the 23S rRNA spacer region (2). PCR amplifications with specific primers for blstTCM, blstSHV, and blstTEM groups 1, 2, 8, 9, 10, and 25, plasmid-mediated blstCMY, sequencing of positive amplicons by primers specific for the blstCMY gene. Salmonella genomic island 1 (SGI1) and class 1 integrons were performed as previously described (11, 14, 15, 18, 22). Positive amplicons were sequenced by Seq Serve, Vaterstetten, Germany.

The results are summarized in Table 1. Plasmid-mediated β-lactamase gene blstCMY, was detected in 3 isolates and blsSHV, 12 in 4. blsCMY, has been reported to occur in Salmonella but has not previously been reported to occur in S. Kentucky isolates from poultry. blsSHV, 12 has been reported to occur in S. Kentucky from a human isolate (7) but not in that from poultry. All isolates contained two plasmids of ca. 4.9 and ca. 130 kb. In all cases, cephalosporin resistance was readily transferable, with cotransfer of both the ca. 4.9-kb and the ca. 130-kb plasmids in every case. blsSHV was detected by PCR in the ca. 4.9-kb plasmid but not in the ca. 130-kb plasmid in isolates with the SHV phenotype, while blsCMY was detected in the ca. 130-kb plasmid but not in the ca. 4.9-kb plasmid in

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isolates with the AmpC phenotype. The associations of bls
SHV-12, with the ca. 4.9-kb plasmid from some isolates but not
other strains of bls CMY-2 with the ca. 130-kb plasmid from
some isolates warrant further analysis, such as PCR-based repli
clon typing (PBRT) with subsequent sequencing, which will
allow us to establish the relatedness of the plasmids identified.
The Salmonella genomic island (SGI-K) was not present, in
contrast to previous reports (15). A class 1 integron was
detected in 1 of 3 CMY-2-positive isolates and in all 4 of the
SHV-12-positive isolates.

The isolates were 92% similar on XbaI PFGE, with sets of 3
(type D) and 2 (type C) isolates indistinguishable. PFGE with
Blnl provided limited additional discrimination between the
isolates. Four isolates were from different broiler farms, with 2
isolates (different PFGE profiles) from the same broiler farm.
One isolate could not be traced. All the broiler farms are
stocked from one breeder farm. Comparison of PFGE patterns
with archived S. Kentucky patterns suggests that this group of
SHV-12 and CMY-2-producing S. Kentucky isolates is closely
related to a number of pan-susceptible S. Kentucky isolates
from human, poultry, and environmental samples (data not
shown).

The simultaneous emergence of broad-spectrum-celahasporin
resistance associated with two distinct plasmid-encoded mechan
isms (ESBL–SHV-12 and AmpC–CMY-2) in poultry in
Ireland as described in this paper is a cause for concern.
Previous experience indicates that such resistant Salmonella
isolates in food animals are to be associated with human dis
ease (4). The route by which these resistant mechanisms gained
access to the multiple poultry farms involved is unclear, but
possibilities include colonization of stock received from
breeder flocks and/or inadequate biosecurity.

It is important to highlight that cephalosporins are not li
censed for use in poultry production in Ireland; however, anoxacillin is used for the control of clostridia and bacterial enteritis and therefore may generate a selective pressure for
possession and retention of a β-lactamase (4, 6, 16), as it is
widely hypothesized that the introduction and persistence of
β-lactamase-producing Salmonella on farms may be sustained
due to selective pressure related to antimicrobial prescribing
(16). However, it has been demonstrated that the acquisition
and persistence of plasmid-mediated β-lactamase in Salmo
nella from commensal intestinal flora can occur even in the
absence of selective antimicrobial pressure (10, 19). The exact
mechanisms of dissemination and maintenance of resistance

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**TABLE 1.** Results

<table>
<thead>
<tr>
<th>Farm</th>
<th>Isolate</th>
<th>Date isolated</th>
<th>Specimen</th>
<th>Antibligram result</th>
<th>PFGE type</th>
<th>Beta-lactamase Type</th>
<th>Class 1 integrin(s)</th>
<th>Variable region(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>08-1060</td>
<td>7 October 2008</td>
<td>Whole chicken</td>
<td>ACACX6FXOCXPd</td>
<td>A</td>
<td>A</td>
<td>CMY-2</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>08-1259</td>
<td>25 November 2008</td>
<td>Chicken neck skin</td>
<td>ACACX6FXOCXPd</td>
<td>B</td>
<td>B</td>
<td>SHV-12</td>
<td>673, 638 aabb, sat</td>
</tr>
<tr>
<td>C</td>
<td>09-0331</td>
<td>16 January 2009</td>
<td>Poultry broth</td>
<td>ACACX6FXOCXPd</td>
<td>C</td>
<td>C</td>
<td>SHV-12</td>
<td>673, 638 sat</td>
</tr>
<tr>
<td>D</td>
<td>09-060</td>
<td>3 February 2009</td>
<td>Whole chicken</td>
<td>ACACX6FXOCXPd</td>
<td>C</td>
<td>C</td>
<td>SHV-12</td>
<td>673, 638 sat</td>
</tr>
<tr>
<td>C</td>
<td>09-061</td>
<td>3 February 2009</td>
<td>Whole chicken</td>
<td>ACACX6FXOCXPd</td>
<td>C</td>
<td>C</td>
<td>SHV-12</td>
<td>673, 638 sat</td>
</tr>
<tr>
<td>E</td>
<td>09-135</td>
<td>18 February 2009</td>
<td>Poultry broth</td>
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<td>D</td>
<td>D</td>
<td>CMY-2</td>
<td>763</td>
</tr>
<tr>
<td>Unknown</td>
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<td>6 March 2009</td>
<td>Whole chicken</td>
<td>ACACX6FXOCXPd</td>
<td>D</td>
<td>D</td>
<td>CMY-2</td>
<td>ND</td>
</tr>
</tbody>
</table>

a For all strains, the SGI was absent, plasmids of ~4.9 and ~130 kb were present, and resistance transfer was observed.

b The values shown indicate the presence of both the 763-bp and the 836-bp variable regions, the 638-bp variable region only, or the 763-bp variable region only. ND, class 1 integron not detected.

Appendix 1
Characterization of a novel extended-spectrum β-lactamase phenotype from OXA-1 expression in Salmonella Typhimurium strains from Africa and Ireland

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Abstract

Salmonella enterica (S. Typhimurium) strains from Kenya, Malawi, and Ireland showing elevated cefepime MIC values carried blaOXA-1. These strains were not detected by current guidelines for extended-spectrum β-lactamase production based on MIC values or clavulanate inhibition, since cefepime is a preferred substrate for OXA-1 and this enzyme is reported to be resistant to clavulanate inhibition. AblaOXA-1 was located within a class I integron and an activated Pr promoter was identified upstream of blaOXA-1. Pr was activated by the insertion of a triplet ‘GGG’ upstream of the −10 signal. All African strains were ST313, prevalent in this continent, and the Irish isolate belonged to ST19.

Keywords: ESBL; Salmonella enterica; Africa; Ireland

Specific detection of extended-spectrum β-lactamase (ESBL) production may become less important for the purpose of treatment due to a consensus to lower the breakpoint for categorization of Enterobacteriaceae as resistant to cephalosporins (CLSI, 2010; EUCAST, 2009). However, detection remains important for infection control and public health purposes (Carmichael, 2004; EUCAST, 2011). There are several phenotypic methods that are available to confirm ESBL production in the microbiology laboratory. All rely on demonstration of synergy of a β-lactam (cefotaxime and cefazidime or cefpodoxime) with clavulanic acid (BSAC, 2008; CLSI, 2007a; Coudron et al., 1997; HPA, 2006).

We have identified a collection of 19 S. Typhimurium that exhibit preferential resistance to cefepime (MIC of 8–32 μg/mL) compared to cefotaxime (MIC of 0.25–2.0 μg/mL) and cefazidime (MIC of 0.5 μg/mL). The cefepime MIC was markedly reduced (0.064–0.125 μg/mL) in the presence of clavulanic acid. The collection comprised 17 clinical isolates collected from blood cultures of adult patients in Nairobi, Kenya (Karuki et al., 2005), 1 isolate from the blood culture of an adult patient from Malawi (PB-1052), and 1 (NSRL-227) isolated from the feces of a patient in Ireland, with associated travel to Andorra (Morris et al., 2006). Details on control bacterial strains utilized for analyses are documented in previous studies published by our group (Boyle et al., 2010; Morris et al., 2003).

Pulsed-field gel electrophoresis (PFGE) was performed using the PulseNet protocol with XbaI and BlnI enzymes (Swaminathan et al., 2001). Analysis of the banding pattern generated was performed using the Dice coefficient with clustering by the unweighted pair group method with arithmetic averaging. Isolates gave distinguishable PFGE...
### Table 1
Extended antibiotic, PCR, sequence analysis, and molecular typing of all ESBL S. Typhirium

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Origin</th>
<th>Resistance profilea</th>
<th>β-Lactamases</th>
<th>Class 1 integrons</th>
<th>2-kb VR</th>
<th>0.8-kb VR</th>
<th>Plasmid profile (kb)</th>
<th>XbaI PFGE</th>
<th>Bgl PFGE</th>
<th>MLST</th>
<th>Sequence type</th>
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<td>Ireland</td>
<td>ACSuTCpOCPcCmRif</td>
<td>OXA-1, TEM-1</td>
<td>1 Class 1 integron (2 kb)</td>
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<td>70, 39</td>
<td>M</td>
<td>H</td>
<td>ST19</td>
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<td></td>
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<td>Malawi</td>
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<td>88, 16.5, 39, 120</td>
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<td>ST313</td>
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<td>ASSwOCPcCmRif</td>
<td>OXA-1, TEM-1</td>
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<td>bβR&lt;sub&gt;NK1&lt;/sub&gt;, aadA4</td>
<td>88, 16.5, 39</td>
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<td>39, 120</td>
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<td>88, 16.5, 39, 105</td>
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<td>F</td>
<td>F</td>
<td>ST313</td>
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</table>

VR= Variable region of class 1 integron.
a= Ampicillin; C= chloramphenicol; S= streptomycin; Su= sulphonamides; T= tetracycline; Cpd= cefpodoxime; Mb= minocycline; Cpo= cefpirome; Cmx= cefoxime; Rif= rifampicin; Na= nalidixic acid; W= trimethoprim; K= kanamycin; Gn= gentamicin.
Table 2
MICs of strains (µg/mL)

<table>
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<th>Isolate no.</th>
<th>MIC (µg/mL)</th>
<th>TZ/ TZL</th>
<th>CT/CTL</th>
<th>PM/ PML</th>
<th>PM</th>
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<td>1.5/0.125</td>
<td>&gt;16/0.19</td>
<td>32</td>
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<td>blaxoxA, blaxoxB</td>
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<td>2/0.125</td>
<td>16/0.19</td>
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<td>0.5/0.25</td>
<td>1/0.194</td>
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<td>blaxoxA, blaxoxB</td>
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<td>&gt;16/0.125</td>
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<td>blaxoxA, blaxoxB</td>
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<td>1/0.194</td>
<td>16/0.19</td>
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<td>16/0.19</td>
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<td>12/0.19</td>
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<td>Top 10 native</td>
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<td>&lt;0.25/0.016</td>
<td>&lt;0.25/0.064</td>
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<td>S. Dublin recipient</td>
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<td>&lt;0.25/0.064</td>
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<td>TCSD pFEP39</td>
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<td>0.50/0.03</td>
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</table>

*Transconjugant Salmonella Dublin with pFEP39 plasmid.
bTop 10 electrocompetent cells with recombinant plasmid pFEP39-1dr conferring phenotype.
XLI0 ultracompetent cells harboring pFEP39-1dr mutant (Δ) with GGG triplet deleted immediately upstream of P1 promoter.

profiles (14 and 9 individual profiles with XbaI and BseH1, respectively). MLST analysis was performed as reported previously (Kidgell et al., 2002; Kingsley et al., 2009) with all African ESBL producers assigned to ST313 and with the Irish ESBL producer assigned to ST19 (Table 1). Therefore, XbaI PFGE results suggest a level of genetic diversity within ST313.

Antimicrobial susceptibility testing, ESBL production, and cefepime MICs were confirmed by Clinical Laboratory Standards Institute (CLSI) methods (CLSI, 2007a,b) and by ESBL Etest method (AB Biodisk, Solna, Sweden). The combination disk method using cefoperazone and cepodoxime plus clavulanic acid failed to confirm ESBL production in study isolates. Four antimicrobial resistance profiles were observed (Table 1). All confirmed ESBL producers were screened by polymerase chain reaction (PCR) for β-lactamase genes using specific primers as reported previously (supplementary Table S1). Isoelectric focusing (IEF) was carried out as previously described (Matthew et al., 1975). The only β-lactamase genes detected by PCR were blaxoxA, blaxoxB, consistent with the detection of 2 β-lactamase enzymes (pl 5.2 corresponding to TEM-1 and pl 7.2 corresponding to OXA-1) on IEF. All 19 isolates harbored a class I integron of 2 kb. Fifteen isolates also harbored a class I integron of 800 bp (Table 1). Plasmid extraction and PCR-based replicon typing (PBRT) were carried out as previously described (Boyle et al., 2010; Carattoli et al., 2005). A common transferable ca. 39-kb plasmid (pFEP39) from all transconjugants was associated with transfer of the ESBL phenotype at 25 °C (Table 2). On PBRT analysis, pFEP39 was identified as a multireplicon plasmid of IncW and H12 types.

BamHI/XbaI-digested pFEP39 fragments were cloned into the pHK-CMV vector (Stratagene, USA) followed by transformation into top 10 E. coli cells. Transformants were selected on cefepime (4 µg/mL) and kanamycin (25 µg/mL) containing LB agar. Sequencing of a 4,937-kb insert (insert ‘1dr’), yielding recombinant plasmid pFEP39-1dr from recombinant clone eNUI-2009 confirmed the β-lactamase blaxoxA as the only β-lactamase present. The cloned 1dr insert was composed of a class I integron with 2 gene cassettes in the variable region, the first being blaxoxA1 with the second cassette being aadA1. A class I integron promoter (Pintegon) upstream of blaxoxA1 was identified. The Pintegon-P1 promoter combination identified in insert 1dr was TGGACA-TAACG (−35/+10 sequence), downstream of which there was a P1 promoter combination of TTGTAA-TACAGT (−35/+10 sequence). P2 had been activated by the insertion of a triplet ‘GGG’
immediately upstream of the –10 signal. The role of this triplet GGG residue on the ESBL phenotype was investigated by site-directed mutagenesis (SDM) using the QuickChange XL SDM kit (Stratagene). The mutated plasmid was named ΔpFEP39-1dr. Targeted deletion of the GGG triplet resulted in a significant change in the ESBL phenotype, with a 32-fold decrease in cefepime MIC observed (Table 2). There is persuasive evidence that the phenotype observed in this study is related to integron-encoded high-level expression of blaOXA-1. A recent study by Jove et al. (2010) investigated the relative strengths of various Psp/P2-P2 promoter combinations of class 1 integrons. The GGG activated P2 promoter was shown to increase expression of the downstream gene cassette (Jove et al., 2010). The 1dr nucleotide sequence data reported in this article will appear in GenBank under accession no. GU119958.

Cepfepime resistance together with moderate resistance to cefotaxime and susceptibility to cefazidime has been reported in association with group III OXA which comprises OXA-1, -4, and -31; however, in all such reports inhibition with clavulanic acid was weak or absent (Dubois et al., 2003; Miro et al., 2002; Oliver et al., 2002).

CLSI recommendations for confirmation of ESBL do not include testing with cepfepime/cefpime–clavulanate acid and are likely to fail to identify isolates with blaOXA-1 in the genetic context described in this study as ESBL producers (BSAC, 2008; CLSI, 2007a; HPA, 2006). The recent CLSI (M100-S20) and European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2011) guidelines state that when using updated criteria (which include revised cephalosporin MICs) it is no longer necessary to carry out routine ESBL confirmation before reporting cephalosporin MICs (CLSI, 2010; EUCAST, 2011). It is appropriate to note that, even with the current lower breakpoints, these isolates would have tested susceptible to cefazidime and susceptible or intermediate to cefotaxime by both CLSI and EUCAST criteria. All but 2 isolates are resistant to cepfepime by EUCAST criteria but most are susceptible or intermediate to cepfepime by CLSI criteria. Given that many such isolates may not be categorized as resistant to commonly used cephalosporins by widely used criteria, it is possible that this pFEP39 plasmid is more widely disseminated but unrecognized.

pFEP39 is present in both multidrug-resistant invasive ST313 S. Typhimurium clonal group and the single S. Typhimurium ST19 clone recovered from an Irish patient following travel to Andorra. ST19 is the most commonly identified Typhimurium sequence type and is recovered globally, whereas ST313 differs only by 1 of 7 alleles from ST19 and has been specifically localized to the sub-Saharan region of Africa (Kingsley et al., 2009). Kingsley et al. (2009) highlighted that ST313 and ST19 S. Typhimurium are circulating simultaneously in Kenya. Cocirculation of 2 sequence types presents the opportunity for genetic exchange and could potentially explain the dissemination of the pFEP39 plasmid among the 2 clonal groups.

In conclusion, further studies are required to determine the extent of geographic dissemination of this resistance phenomenon in S. enterica and to determine whether the plasmid has disseminated into other Enterobacteriaceae.

Supplementary materials related to this article can be found online at doi:10.1016/j.diagmicrobio.2011.04.007.

Acknowledgments

The authors acknowledge the contribution of the late Prof. Tony Hart to this work.

Positive control strains harboring blaCTX-M-15, blaCTX-M-16, and blaOXA-1 were kindly provided by Prof. Peter Hawkey, University of Birmingham, UK, and by Dr. Neil Woodford, Health Protection Agency, UK. Positive controls for PBRT were kindly provided by Dr. Alessandra Carattoli, Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy. Positive controls for Salmonella genomic island were provided by Ciara Walsh, Center for Food Safety, UCD Veterinary Sciences Center, University College Dublin, Ireland. The authors would also like to thank Prof. Mark Achtman, Environmental Research Institute, University College Cork, Cork, Ireland, for his help with MLST analysis and for curating the Salmonella enterica MLST website and database.

References


*Genome Res* 19:2279–2287.


Research letters

HVH, were assigned to the novel sequence type designated ST-326, and five isolates from HVH to the novel sequence type ST-327. The third clonal cluster, comprising five isolates from HSP and seven isolates from HVH, was identified as the ST-1 strain. Notably, isolates from ST-1 were found to persist at least from 2005 to 2007, and isolates from ST-326 and ST-327, from 2007 to 2008. Moreover, representatives of ST-1 and ST-326 were detected in two different hospitals each, with ST-326 causing an outbreak at one of these. The remaining four isolates represented the previously defined sequence types ST-147, ST-37, ST-14 and ST-321 (Table 1).

The oxacillinase-encoding gene blaoxa-1 and the bifunctional acetyltransferase-encoding gene aac(6'')-Ib-cr have been detected previously in CTX-M-15-producing strains of E. coli and K. pneumoniae. In our study, the blaoxa-1 and aac(6'')-Ib-cr genes were detected in all ST-326 isolates (pulsotype I) and in all but three ST-1 isolates (pulsotypes VII and VIII). Additionally, all ST-326 isolates (pulsotype I) but one also carried the wild-type gene aac(6'')-Ib-cr. In contrast, only one of the ST-327 isolates (pulsotype IV) carried the aac(6'')-Ib-cr gene (Table 1).

Moreover, we found that all isolates from ST-326 and ST-1 exhibited high-level resistance to quinolones and florfenicolokes. Those isolates showed quinolone resistance-associated predicted amino acid changes within the QRDR of both the gyrA and parC genes. Notably, within the total population, 26 of the 29 quinolone-resistant isolates carried the gyrA- and parC- QRDR-cr gene, whereas this gene was absent from all (but one) of the isolates susceptible to quinolones and florfenicolokes (Table 1). Since all study isolates were PCR-negative for the qnr and qepA genes, these results are consistent with the previously described involvement of the AAC(6'')-Ib-cr enzyme in the selection for QRDR chromosomal mutations, resulting in clinically relevant quinolone resistance levels.

In conclusion, our findings document the dissemination and persistence of K. pneumoniae clonal strains producing CTX-M-15 in different hospitals within the Barcelona metropolitan area. Two of these clones, i.e. ST-326 and ST-1, exhibited resistance to aminoglycosides and quinolones and were disseminated to at least two hospitals each. This report provides the first description of an outbreak caused by such strains within our country. These results may indicate that the observed high frequency of hospital outbreaks mediated by these strains may be a consequence of the strains’ persistence within the affected locale.

Acknowledgements

We are grateful to Sylvain Brisse (Génotypage des pathogènes et Santé publique, Institut Pasteur, Paris, France) for validating and assigning designations to the new MLST profiles found in this study, and to P. Courvalin (Institut Pasteur, Paris, France) for providing E. coli TOP10/pRTH51 harbouring qepA.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


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CTX-M enzymes are the predominant extended-spectrum β-lactamases produced by Enterobacteriaceae in Ireland

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Appendix 3

Research letters

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Keywords: antimicrobial resistance, cefotaxime, plasmid-mediated

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Sir,

Several recent reviews have detailed how CTX-M β-lactamases have become dominant among extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (ESBL-PE) in many European countries. However, data from Ireland were lacking. This study represents a nationwide survey of ESBL-PE collected throughout Ireland over an 11 year period and highlights the predominance of CTX-M-producing strains.

From 1997 to 2010 inclusive, 812 isolates of Enterobacteriaceae were received from 25 clinical laboratories (A–Y) throughout Ireland, with 761 isolates submitted by individual laboratories, predominantly urinary origin (259). ESBL production was confirmed by CLSI disc diffusion and/or Etest in 506 (62%) isolates from 462 patients from 18 clinical laboratories (1–332 ESBL-PE per laboratory) (Table S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org)). All analyses were performed on the first isolate received per patient. The 462 isolates were predominantly Escherichia coli (n = 391, 84.5%) and from urine (n = 318, 68.8%) (Table S2, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org)). Antimicrobial susceptibility testing revealed that all ESBL-PE were resistant to >1 antimicrobial agent, with 75% resistant to >8 of 16 antimicrobials tested. Of particular concern is the high proportion of ESBL-PE co-resistant to nalidixic acid (82%), ciprofloxacin (73%), trimethoprim (69%) and gentamicin (38%), as these are frequently important alternatives to β-lactams for the treatment of various infections (Table S3, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org)). This pattern of co-resistance has been reported in other countries.

PCR revealed that of the 462 isolates, 371 (80%) isolates harboured blaCTX-M (222 (60%) harboured a blaCTX-M-group-1 gene and 149 (40%) harboured a blaCTX-M-group-9 gene), 280 (65%) harboured blaTEM and 81 (18%) harboured blaseve (Table S4, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org)). Among 348 CTX-M-producing E. coli, PFGE analysis using XbaI identified 126 individual pulse-field profiles (PFPs) and 35 clusters based on a similarity of ≥85%. These clusters comprised 2–37 members. All members of each clonal group carried blaCTX-M genes belonging to the same phylogenetic group. Overall, 173 (50%) of 348 isolates belonged to one of six major clusters (groups 1–6 comprising 13–37 members from 1–8 clinical laboratories) (Table 1 and Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org)). Group 4 included 23 isolates collected in the course of an investigation of an outbreak in a nursing home. The UK epidemic 'strain A' clustered with 'group 1' in this study (Figure S1), indicating that this clonal group has disseminated widely in Ireland.

Denaturing HPLC (dHPLC) analysis was performed on 34 blaCTX-M-positive isolates representative of 29 PFPs, which represent 254 (50%) of all ESBL-PE. Twenty-two isolates representative of 17 PFPs

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Table 1. Correlation of each of the six major PFGE groups of CTX-M-producing E. coli identified with antibiogram, bla-type, dHPLC and sequence analysis.

<table>
<thead>
<tr>
<th>PFGE group</th>
<th>No. of isolates per group</th>
<th>No. of referring clinical laboratories</th>
<th>No. of individual antibiograms</th>
<th>No. of isolates per PF group</th>
<th>No. of PFGE profiles (PFPs)</th>
<th>No. of clusters of isolates</th>
<th>No. of isolates per cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>35</td>
<td>35</td>
<td>8</td>
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<td>2</td>
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<td>15</td>
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<td>15</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

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865

170
Supplementary data

Tables S1–S4 and Figure S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org).

References


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Plasmid-mediated 16S rRNA methylases among extended-spectrum β-lactamase-producing Salmonella enterica Senftenberg isolates from Algeria

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Keywords: ESBLs, nosocomial, CTX-M-3, ArmA

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Transparency declarations

None to declare.
Appendix 4

Enumeration and Characterization of Antimicrobial-Resistant *Escherichia coli* Bacteria in Effluent from Municipal, Hospital, and Secondary Treatment Facility Sources

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Received 1 December 2009/Accepted 18 May 2010

We describe a modification of the most probable number (MPN) method for rapid enumeration of antimicrobial-resistant *Escherichia coli* bacteria in aqueous environmental samples. *E. coli* (total and antimicrobial-resistant) bacteria were enumerated in effluent samples from a hospital (n = 17) and municipal sewers upstream (n = 5) and downstream (n = 5) from the hospital, effluent samples from throughout the treatment process (n = 4), and treated effluent samples (n = 13). Effluent downstream from the hospital contained a higher proportion of antimicrobial-resistant *E. coli* than that upstream from the hospital. Wastewater treatment reduced the numbers of *E. coli* bacteria (total and antimicrobial resistant); however, antimicrobial-resistant *E. coli* was not eliminated, and *E. coli* resistant to cefotaxime (including extended-spectrum beta-lactamase [ESBL] producers), ciprofloxacin, and ceftoxitin was present in treated effluent samples.

The emergence and dissemination of antimicrobial resistance are well established as clinical problems that affect human and animal health. *Escherichia coli* is an important element of the flora of the human and animal intestine and a significant pathogen associated with gastrointestinal infection, urinary tract infections, and a variety of other extraintestinal infections (4). *E. coli* shed into the environment can survive for significant periods (7, 14, 23). Detection of *E. coli* in water and food is widely used as a microbiological indication of fecal contamination.

Data on the significance of environmental contamination with antimicrobial-resistant *E. coli* for human health are limited. Previous reports have shown that antimicrobial-resistant strains of bacteria are present in various effluents, such as hospital effluent discharge (8, 10, 16, 21), inflow effluent to a wastewater treatment plant (WWTP) (15), and outflow-treated effluent from a wastewater treatment plant (2, 12, 13, 18, 27).

A wastewater treatment plant treating effluent from hospitals may be associated with discharge of relatively high levels of antimicrobial-resistant *E. coli* compared with those of a plant treating municipal effluent that does not include hospital effluent discharge (22). There are few reports of quantitative data on antimicrobial-resistant *E. coli* bacteria in effluent, reflecting the lack of a convenient method for their enumeration (12, 15, 22). Previous methods available for the detection of antimicrobial-resistant *E. coli* in a water sample have generally involved the isolation of *E. coli* and the selection of some isolates for susceptibility testing. In such cases, the proportions of antimicrobial-resistant organisms are based only on those isolates selected and are therefore not representative of the entire population. By adding the antimicrobial agent of interest to the water sample before testing, we have adapted a commercial most probable number (MPN) method (the Colilert system) for enumerating the total number of *E. coli* isolates resistant to that agent in a sample.

MATERIALS AND METHODS

Validation of the method with simulated samples. For validation of the method, suspensions of *E. coli* ATCC 25922 (susceptible to all tested agents) and of antimicrobial-resistant clinical *E. coli* strains (Table 1) were prepared to equal a 0.5 McFarland standard (1.5×10^6 CFU/ml). Dilutions were prepared in sterile distilled water to give suspensions of *E. coli* within the enumeration range of the Colilert Quanti-Tray 2000 (IDEXX, Technopat, Limerick, Ireland) (<2419.6 MPN/100 ml). Suspensions of *E. coli* ATCC 25922 alone and mixed with defined proportions of antimicrobial-resistant clinical strains were prepared. Total *E. coli* bacteria enumeration was performed on a 100-ml aliquot of suspension using Colilert Quanti-Trays (IDEXX, Technopat, Limerick, Ireland), according to the manufacturer’s instructions.

Stock solutions of ampicillin, streptomycin, sulfamethoxazole, tetracycline, cefoxitin, cefotaxime, and ciprofloxacin (Sigma, Dublin, Ireland) were prepared. A volume of stock antimicrobial solution was added to a 100-ml aliquot of the aqueous sample to achieve the required final concentrations of ampicillin (32 μg/ml), streptomycin (32 μg/ml), sulfamethoxazole (256 μg/ml), tetracycline (4 μg/ml), cefoxitin (32 μg/ml), cefotaxime (2 μg/ml), and ciprofloxacin (4 μg/ml). The concentration of cephalosporin was selected to facilitate detection of extended spectrum beta-lactamase (ESBL)-producing *E. coli*, for which elevation of the cefotaxime MIC may be modest (3). Following addition of the antimicrobial agent, the specimen was processed in Colilert Quanti-Trays, according to the manufacturer’s instructions. Following incubation at 37°C (±1°C) for 18 to 24 h, trays were read, according to the manufacturer’s instructions. The backs of the trays were disinfected with 70% ethanol, and 1-ml aliquots were taken using a sterile syringe and needle (Sartdol, Nümbrecht, Germany). *E. coli* was isolated from these aliquots and identified using API 20E (bioMérieux, Inc., Marcy I’Etoile, France). Antimicrobial susceptibility testing was performed by using Clinical Laboratory Standards Institute (CLSI) disc diffusion methods to confirm that positive wells reflected growth of targeted antimicrobial-resistant *E. coli* (3).

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TABLE 1. Composition of antimicrobially contaminated samples (E. coli strains/phenotypes) for validation of method and results obtaineda

<table>
<thead>
<tr>
<th>E. coli in suspension</th>
<th>Antimicrobial agent (amt [µg/ml])</th>
<th>No. of resistant E. coli strains (MPN/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 25922</td>
<td>None</td>
<td>344</td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>Cefotaxime (2)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>ATCC 25922</td>
<td>None</td>
<td>816</td>
</tr>
<tr>
<td>ATCC 25922 and 33β</td>
<td>Cefotaxime (2)</td>
<td>365</td>
</tr>
<tr>
<td>ATCC 25922 and 25γ</td>
<td>None</td>
<td>709</td>
</tr>
<tr>
<td>ATCC 25922 and 25γ</td>
<td>Cefotaxime (2)</td>
<td>570</td>
</tr>
<tr>
<td>ATCC 25922 and 22β</td>
<td>None</td>
<td>698</td>
</tr>
<tr>
<td>ATCC 25922 and 22β</td>
<td>Ciprofloxacin (4)</td>
<td>308</td>
</tr>
</tbody>
</table>

a The artificially contaminated samples were constituted to have approximately 300 CFU/100 ml of each strain present in each sample (total of c. 600 CFU in samples containing 2 strains).

b 33, cefotaxime-resistant clinical strain of E. coli (2 µg/ml) harbors blaTEM, blafloxacin-resistant clinical strain of E. coli (resistance mechanism characterized).

c 27, cefotaxime-resistant clinical strain of E. coli (resistance mechanism characterized).

d 22β, ciprofloxacin-resistant clinical strain of E. coli (>32 µg/ml, ciprofloxacin resistance mechanism characterized, que negative) harbors blaTEM, blafloxacin-resistant clinical strain of E. coli (resistance mechanism characterized).

RESULTS

Validation of method. (a) Artificially contaminated samples. The numbers of antimicrobial-susceptible and antimicrobial-resistant E. coli strains in each artificially contaminated sample are summarized in Table 1. The results confirm that only the antimicrobial-resistant population of E. coli is enumerated in the presence of the relevant antimicrobial agent. Testing on isolates from the antimicrobial-containing trays confirmed that the E. coli isolates growing in the tray were the antimicrobial-resistant isolates. In artificially contaminated samples, ciprofloxacin-resistant E. coli strain 22β was detected in suspensions, in which it represented as little as 0.0001% of the total E. coli population (approximately 15 CFU of E. coli 22β mixed with 1.5 × 10^7 E. coli ATCC 25922).

(b) Test samples. (a) Hospital effluent discharge. Data are summarized in Table 2. Amoxicillin-resistant E. coli was detected in every sample of hospital A effluent and was generally present in higher proportions than other antimicrobial-resistant E. coli, accounting for 100% of the total population in one sample (HAI9). E. coli isolates resistant to streptomycin, sulfamethoxazole, and tetracycline were also present at relatively high levels in most samples. E. coli isolates resistant to the cephalosporins and ciprofloxacin were present less consistently and generally at lower levels. A higher proportion of E. coli resistant to ampicillin is correlated to a high proportion of E. coli resistant to streptomycin (r = 0.8; P = 0.001). No other correlation between the proportions could be observed.

(c) Municipal effluent. The number of E. coli isolates in samples of effluent downstream from hospital A was significantly reduced in comparison with the number of those isolates in samples of effluent taken upstream (P = 0.047). In municipal effluent downstream from the hospital effluent discharge, the proportion of E. coli resistant to all tested antimicrobial agents was considerably higher than the proportion of that in the upstream samples, but this reached borderline statistical significance for only one antimicrobial agent (sulfamethoxazole) (P = 0.047).

(c) Effluent from wastewater treatment plant. The number of E. coli isolates (MPN/100 ml) in the treated effluent sample ranged from 5.76 × 10^5 to 1.55 × 10^5, representing an approximately 1,000 (3-log_10) decrease from those in municipal and hospital effluent discharge samples; however, a large propor-
<table>
<thead>
<tr>
<th>Location</th>
<th>Sample code</th>
<th>Sample date (day/mo/yr)</th>
<th>No. of E. coli strains (MPN/100 ml)</th>
<th>% of antimicrobial resistance</th>
<th>A</th>
<th>S</th>
<th>Su</th>
<th>T</th>
<th>F</th>
<th>P</th>
<th>X</th>
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<tbody>
<tr>
<td>Efluent upstream from hospital A</td>
<td>UA 01</td>
<td>15/11/2006</td>
<td>$3.01 \times 10^7$</td>
<td>1.4</td>
<td>1.4</td>
<td>15.9</td>
<td>2.9</td>
<td>1</td>
<td>0</td>
<td>0.03</td>
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<tr>
<td></td>
<td>UA 02</td>
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<td>7.6</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>UA 03</td>
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<td>$1.1 \times 10^7$</td>
<td>12.4</td>
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</tr>
<tr>
<td></td>
<td>UA 04</td>
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<td>$2.0 \times 10^7$</td>
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<tr>
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<td>Mean</td>
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<td>$1.5 \times 10^7$</td>
<td>7.72</td>
<td>7.81</td>
<td>5.62</td>
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<td>0.17</td>
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<td>Hospital A effluent</td>
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<td>$6.38 \times 10^6$</td>
<td>32</td>
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<td>FTE 07 (pm)</td>
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<td></td>
<td>FTE 08 (pm)</td>
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</table>

* A = ampicillin; S = streptomycin; Su = sulfamethoxazole; T = tetracycline; F = cefotaxime; P = ciprofloxacin; X = coliform; NT = not tested.
* UA = upstream from hospital A; HA = hospital A; DA = downstream from hospital A; RIE = raw intake effluent; PTE = primary-treated effluent; PRT = post-return effluent; AE = aeration effluent; FTE = final treated effluent.
tion of E. coli resistant to each antimicrobial agent was detected in the treated effluent sample.

Analysis shows that total E. coli isolate counts at consecutive sampling points through the treatment process show a significant decline (linear $R^2 = 80.6%$; $P = 0.039$). This decline was observed in the following order: raw intake effluent (stage 0), post-return effluent (stage 1), primary-treated effluent (stage 2), aeration effluent (stage 3), and final treated effluent (stage 4). The percentage of antimicrobial-resistant E. coli in effluent at sampling points throughout the treatment process showed no consistent pattern of increase or decrease using linear regression analysis.

As the numbers of effluent samples taken from throughout wastewater treatment were limited, these results are indicative rather than conclusive.

Characterization of antimicrobial-resistant isolates. After enumeration of total E. coli bacteria in effluent samples, 254 isolates were collected from effluent samples from individual Colilert Quanti-Tray wells (Table 3). All isolates were resistant to the antimicrobial agent used in the corresponding Colilert Quanti-Tray, thus confirming the specificity of the method for isolation of the targeted antimicrobial-resistant E. coli isolates. Isolates were obtained from hospital A effluent samples ($n = 106$), municipal effluent samples upstream ($n = 36$) and downstream ($n = 36$) from hospital A, treated effluent samples (2006 and 2007) ($n = 47$), and effluent samples from throughout the treatment process ($n = 29$). These isolates were obtained from trays selecting for resistance to ampicillin ($n = 60$; 24%), streptomycin ($n = 44$; 17%), sulfamethoxazole ($n = 44$; 17%), tetracycline ($n = 40$; 16%) cefotaxime ($n = 19$; 7%), cefoxitin ($n = 12$; 5%), and ciprofloxacin ($n = 35$; 14%). As expected, a high correlation was observed among resistance to antimicrobials of the same class, e.g., nalidixic acid and ciprofloxacin. In some instances, correlations were also observed among resistances to antimicrobial agents of different classes, in particular, for ampicillin, streptomycin, and sulfonamide. Screening with cefotaxime and ciprofloxacin selected for E. coli isolates with overall higher levels of resistance to all antimicrobials tested than screening with older antimicrobial agents (Table 3).

Detection of extended-spectrum beta-lactamase producers. Twenty-three (22) extended-spectrum beta-lactamase (ESBL)-producing E. coli isolates were identified from the 254 total isolates studied (Table 4). These isolates were obtained from hospital A effluent samples ($n = 5/106$) and municipal effluent samples upstream ($n = 1/36$) and downstream ($n = 1/36$) from hospital A. From the 47 E. coli isolates obtained from treated effluent samples, one ESBL-positive isolate was obtained from the intake effluent of a treatment plant, seven were obtained from secondary-treated effluent (2006 samples), five were obtained from the secondary-treated effluent (2007 samples), one was obtained from primary-treated effluent (2008 sample), and two were obtained from secondary-treated effluent (2008 sample). The number of ESBL-producing E. coli isolates obtained from treated effluent ($n = 15$; 6%) was higher than those obtained from nontreated effluent ($n = 8$; 3%). However, as the total number of ESBL-producing isolates was not enumerated, and the isolates for characterization were selected in an unstructured way, statistical analysis to assess the apparent difference was not carried out.
Appendix 4

TABLE 4. Molecular characterization of ESBL-producing *E. coli* isolates from effluent samples

<table>
<thead>
<tr>
<th>Isolate origin</th>
<th>Specimen no.</th>
<th>Sample code</th>
<th>Antimicrobial resistance profilea</th>
<th>Detection of antimicrobial resistance genes</th>
<th>PFGE profile (&lt;85%b)</th>
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<td></td>
<td></td>
<td></td>
<td><em>bla</em>&lt;sub&gt;TEM&lt;/sub&gt;</td>
<td><em>bla</em>&lt;sub&gt;SHV&lt;/sub&gt;</td>
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<td>+</td>
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<td>ATKFFZD</td>
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<td>-</td>
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<tr>
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<td>ASuRKKPFZD</td>
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<td>ARNKPFZD</td>
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<td>+</td>
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</tr>
<tr>
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<td>FTE 10</td>
<td>ASF</td>
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a. A = ampicillin; C = chloramphenicol; S = streptomycin; Su = sulfamethoxazole; T = tetracycline; R = trimethoprim; N = nalidixic acid; K = kanamycin; P = piperacillin; F = cephalaxin; Z = oxazolidine; X = ceftoxin; M = minocycline; G = gentamicin; D = cephalodoxin.

b. Letter assignment based on >85% similarity with banding patterns.

c. DA = downstream from hospital A.

d. UA = upstream from hospital A.

The *bla<sub>CTX-M</sub>* gene was present in 22 of 23 ESBL-producing isolates, with all belonging to group 1 (n = 19; 86%) or group 9 (n = 3; 14%) *bla<sub>CTX-M</sub>*. Sequencing of *bla<sub>CTX-M</sub>* genes from samples of treated effluent discharged into the environment identified group 1 genes as *bla<sub>CTX-M-28</sub>* (n = 1), *bla<sub>CTX-M-3</sub>* (n = 2), *bla<sub>CTX-M-61</sub>* (n = 3), and *bla<sub>CTX-M-15</sub>* (n = 2) and the group 9 gene as *bla<sub>CTX-M-14</sub>* (n = 3). The *bla<sub>TEM</sub>* gene was detected in 11 isolates in the following samples: hospital A effluent (n = 1), raw intake effluent (n = 1), primary-treated effluent (n = 1), and secondary-treated effluent (n = 8). The *bla<sub>SHV</sub>* gene was detected in one hospital A effluent sample isolate (Table 4).

**Pulsed-field gel electrophoresis (PFGE) of ESBL producers.**

PFGE analysis showed considerable diversity of genotypes among the isolates examined (Fig. 1). Indistinguishable pulsed-field profiles were obtained for isolates 0108 and 0121, obtained from final treated effluent samples on different dates; however, the isolates differed in antimicrobial resistance pattern (Table 4). Both isolates harbored the *bla<sub>TEM</sub>* and *bla<sub>CTX-M-14</sub>* genes.

PFGE-indistinguishable isolates 0895 and 0900, both obtained from secondary-treated effluent samples collected at different times on the same day in 2007, were resistant to ampicillin, streptomycin, sulfonamide, trimethoprim, nalidixic acid, ceftoxime, and cefotaxime. Isolate s-0895 was also resistant to ciprofloxacin, with a MIC of >32 μg/ml, while isolate s-0900 was intermediate to ciprofloxacin, with a MIC of 2 μg/ml. Both isolates harbored *bla<sub>CTX-M-61</sub>* genes. A third isolate, 0891 (from the same sample as 0895), also obtained from treated effluent samples, was very similar (97.5%) to isolates 0895 and 0900 by PFGE but with additional resistance to minocycline. All isolates were negative for *qnrA*, *qnrB*, and *qnrS*.

Isolates 0982 and 1016 were obtained from samples of municipal effluent near hospital A (one upstream and one downstream). These isolates showed 97.5% similarity of PFGE pattern by BioNumerics analysis.

A comparison of pulsed-field profiles from effluent isolates and ESBL-producing *E. coli* clinical isolates (as previously described [17]) was performed. All clinical isolates were obtained from samples received in the same hospital from which effluent samples were collected. Overall, the resulting dendrogram shows that most clinical isolates cluster together with more than 75% similarity, with only a small number of effluent isolates within this group. Most effluent isolates are relatively distinct from clinical isolates and from each other, although a small number of clinical isolates are grouped among the predominantly environmental clusters (Fig. 1). The largest cluster of isolates (n = 27) all harboring group 1 *bla<sub>CTX-M</sub>* genes comprises 25 clinical isolates and 2 isolates from hospital A effluent samples (0586 and 0977). By PFGE, effluent isolate 0586 was 97.5% similar to clinical isolate c-838 obtained from a urine sample. Isolate 0977 showed 97.5% similarity to four clinical isolates (c-918, c-900, c-690, and c-901) obtained from urine samples. It is of note that the two environmental isolates (0586 and 0977) most similar to clinical isolates were directly from samples of hospital effluent discharge rather than those taken from other sites. However, there are limitations to the
FIG. 1. Pulsed-field gel electrophoresis dendrogram of extended-spectrum beta-lactamase producers from effluent and clinical sources. UA = upstream from hospital A; HA = hospital A effluent; DA = downstream from hospital A; RIE = raw intake effluent; PTE = primary-treated effluent; FTE = final treated effluent.
typing methods applied, and conclusions regarding relationships between isolates are tentative.

**DISCUSSION**

The MPN method is an established approach to enumeration of *E. coli* in bacteria in water. Colilert products are widely used for this purpose because they are simple to use and interpret. We have demonstrated that the Colilert system can be readily adapted for enumeration of *E. coli* bacteria resistant to specific antimicrobial agents in aqueous samples. As a result, the proportion of antimicrobial-resistant *E. coli* bacteria in an aqueous environmental sample can be calculated. Previous methods have generally involved studying selected isolates obtained from filtration of aqueous samples. The method described here can be used for detection of extended-spectrum beta-lactamase (ESBL)-producing organisms, in addition to other specific resistance phenotypes. The method can also be applied to enumeration of *E. coli* bacteria with specific multiple antimicrobial resistance phenotypes by addition of multiple antimicrobial agents (data not shown).

Antimicrobial-resistant bacteria may be discharged into the environment from human sources (hospital and municipal effluent) and agricultural sources (15, 16). There is considerable potential for dissemination of antimicrobial-resistant organisms and resistant determinants from such sources through contamination of food and water (5, 11). The risk may be greatest when contaminated wastewater is discharged directly into the environment. However, secondary wastewater treatment does not remove all fecal organisms. The Environmental Protection Agency (EPA) in Ireland estimates that approximately 70% of wastewater in Ireland is subject to secondary treatment (19). Outflow from secondary wastewater treatment facilities has much reduced *E. coli* levels compared with those of untreated effluent; however, there are limited data to indicate the extent to which antimicrobial-resistant *E. coli* bacteria are removed through the treatment process and the levels present in the discharge. Our data indicate that a high proportion of *E. coli* discharged from secondary wastewater treatment facilities is resistant to ampicillin and that isolates resistant to newer agents such as extended-spectrum cephalosporins (including the ESBL phenotype) and fluoroquinolones are also present. It appears that antimicrobial-resistant *E. coli* is not at any significant survival disadvantage in the environment.

This is the first report confirming that ESBL-producing *E. coli* survives the wastewater treatment process of a modern secondary treatment facility. ESBL-producing *Escherichia coli* has emerged as a significant human health issue in hospitals and communities in the past decade. It is a significant local and global health concern (9, 17, 25, 29). We have previously documented an outbreak of ESBL-producing *E. coli* in a nursing home in this region, and infection with ESBL-producing *E. coli* is now a common clinical problem (17, 20). The ESBL variants (CTX-M groups 1 and 9) detected in the outflow of the wastewater treatment plant generally correspond to the most common variant/variants detected in association with clinical infection in our population (17). Pulsed-field gel electrophoresis shows considerable heterogeneity among the environmental ESBL-producing *E. coli* bacteria isolated, and they are frequently quite different from isolates associated with human infection by PFGE. However, three closely related strains harboring bluCTX-M-61 (isolates #81, 895, and 900) were isolated from two effluent samples taken on the same day (15 August 2007). This CTX-M variant has not previously been reported in Ireland (17). The diversity in CTX-M variants detected in the treated effluent samples reflects the spread of these important genotypes in the environment.

The comparison between clinical and environmental ESBL-producing *E. coli* shows that environmental isolates similar to clinical isolates were detected in direct hospital effluent discharge samples on two occasions. One might speculate that PFGE diversity of ESBL-producing *E. coli* in the wider environment may be related to plasmid transfer between *E. coli* strains in the environment and or because those isolates associated with clinical infection are a virulent subset of ESBL-producing *E. coli* associated with gastrointestinal colonization.

The extent to which discharge of antimicrobial-resistant *E. coli* and other bacteria into the environment contributes to the dissemination of antimicrobial resistance is uncertain. To date, studies of antimicrobial resistance in bacteria in the environment have tended to address the issue in terms of the presence or absence of antimicrobial-resistant bacteria (1, 6, 21). Quantitative data are likely to be important in efforts to assess the potential risk and to assess the impact of specific elements of the effluent treatment process in removing antimicrobial-resistant *E. coli*, as quantitative microbial risk assessment is increasingly being used. The method we report is a convenient method for enumeration of antimicrobial-resistant *E. coli* bacteria in aqueous samples and will be valuable in facilitating more detailed studies of the potential impact of municipal and agricultural effluent on antimicrobial resistant *E. coli* in the environment.

**ACKNOWLEDGMENT**

This research is funded by the Environmental Protection Agency (EPA) of Ireland, through the national development plan, as part of the Enhancing Human Health Through Improved Water Quality (EHHTIWQ) project (http://www.mnaighway.ie/chw).

**REFERENCES**


Appendix 4

Vol. 76, 2010


Title: Enterococcus faecium of vanA genotype in rural drinking water, effluent and the aqueous environment

Running title: VRE in the aqueous environment

Authors: Dearbhaille Morris¹, Sandra Galvin¹, Fiona Boyle¹, Paul Hickey², Martina Mulligan³ and Martin Cormican⁴

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Keywords: Vancomycin resistant enterococci, environment, water, effluent

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Abstract:

Total enterococci and VRE were enumerated in samples of effluent (n=50) and water (n=167) from a number of sources. VRE were detected in the outflow of a wastewater treatment plant and in a single rural drinking water supply suggesting potential for transmission to humans through environmental contamination.
Enterococci with acquired high level (≥ 256 μg/ml) resistance to vancomycin were first reported in Europe in 1988, and have since been reported worldwide with increasing frequency (10). In Ireland 44% of invasive isolates of Enterococcus faecium were vancomycin resistant in the second quarter of 2011. [http://www.hpsc.ie/hpsc/A-Z/MicrobiologyAntimicrobialResistance/EuropeanAntimicrobialResistanceSurveillanceSystem/EARSS/EARSSSurveillanceReports/2011Reports/File.12962.en.pdf].

Environmental contamination is a factor in dissemination of VRE in hospitals (13) however the role of contamination of the general environment is less well explored. It has been reported that enterococci can persist for long periods in the environment (2, 5).

This is the first report on the occurrence of VRE in waters and effluents from a number of sources in Ireland.

Fifty samples of effluent were collected between 2006 and 2008: hospital effluent (Hospital A (HA) (n=17); Hospital B (HB) (n=2)), municipal effluent upstream and downstream from the effluent discharge points of Hospital A (USA, n = 5; DSA, n = 5), and Hospital B (USB, n=2 ; DSB, n=2); final treated effluent from a secondary wastewater treatment process (TE, n=13) and on a single occasion in 2008 from a number of points throughout the wastewater treatment process (n=4). Seawater (n=29), river (n =28) and lake water samples (n=8) were collected in 2006 and 2007. Source (S, n=17) and distributed (P, n=17) water samples were taken from 3 rural group water supplies, K, M, and C between October 2006 and June 2007.
Total enterococci were enumerated using the Enterolert® Quanti-tray® system (IDEXX, Technopath, Limerick) (6) and the proportion of VRE was determined by addition of vancomycin to a final concentration of 256μg/ml. Effluent from Hospital A contained the highest numbers of enterococci (up to 1.1 x 10^7 MPN/100ml detected (Table 1) and percentage (0.02 to 27%) of VRE (Table 1). VRE were also detected in 2 of 5 (40%) samples of municipal effluent downstream of Hospital A, in 2 of 13 (15%) samples of final treated effluent, in 1 source water sample from K supply and in a single river water sample (Table 1).

Aliquots (1ml) were removed from one positive well per tray as previously described (6) leading to the collection of 20 isolates of VRE (HAS, n=13; DSA, n=2; TE, n=3; KS, n=1; and urban river water, n=1). All were identified as Enterococcus faecium and had vancomycin MICs of >256μg/ml as determined by Etest® (BioMérieux Inc., Marcy l’Etoile, France), validating the modification of the Enterolert® Quanti-tray® system for detection of VRE. The vanA gene was confirmed in all isolates by PCR (4). Pulsed field gel electrophoresis (PFGE) analysis using Smal identified 18 major pulsed field profiles (PFPs) and 5 major clusters (Cluster A to E) containing 2-7 isolates based on a PFP similarity of ≥ 85% (Figure 1). The 10 blood stream isolates of VRE included 9 distinct PFPs and were not similar to environmental isolates (Figure 1).

Caplin et al. (2008) reported high level (≥128μg/ml) VRE in 4/26 (15%) hospital effluent samples and in 15/21 (71%) of untreated municipal effluent samples in England (3). Kuhn et al. (2005) reported VRE in 36% of urban wastewater, and 16% of hospital
Appendix 5

94 effluent samples examined from 4 countries (Sweden, Spain, United Kingdom and Denmark) (7). Novais et al. (2005) reported detection of VRE in 11/14 (79%) of wastewater samples downstream from 4 hospitals in Portugal (12). We have evaluated VRE as a percentage of total enterococci and demonstrate that VRE are not only present frequently in hospital effluent but also in high numbers compared with previous findings (4, 10, 16). The influence of hospital effluent on the presence of VRE in municipal effluent downstream from the hospital is also clearly demonstrated.

97 While VRE appears to be partly removed by wastewater treatment VRE were detected in two treated effluent samples (2% of enterococci detected). Arasijo et al. (2010) reported no significant differences in the proportion of VRE between the inflow and effluent from wastewater treatment plants (1). Vilanova et al. (2004) also found that VRE persisted after the treatment process (14).

99 Lanthier et al. (2010) reported that enterococci resistant to ciprofloxacin (2 μg/ml) or vancomycin (16 μg/ml) were uncommon in a river basin in Canada (8). Novais et al (2005) found VRE (all E. faecium of the vanA genotype) in 2/3 samples collected from a river in Portugal (12). Moore et al (2008) reported no VRE in surface and ocean waters from California (11). In these reports vancomycin at 16 μg/ml was used for screening compared with 256 μg/ml in this study. The higher concentration used here excludes detection of low level intrinsic resistance. Our data indicate that VRE is infrequently detected in river (1/28), lake (0/8) and seawater (0/29). This is the first report of vanA VRE in a drinking water supply. Although detection of VRE in 1 of 51 drinking water
Appendix 5

117 sources seems a low frequency it is worth noting that this represents detection once in a
total volume of just 5.1L. The situation in Ireland whereby some rural dwellers are
served by small water supply systems with limited or inconsistent treatment also pertains
in rural areas of a number of other developed countries.

121 PFGE does not demonstrate a close relationship between the clinical and environmental
isolates of VRE. The clinical isolates were blood stream isolates. Studies have
demonstrated that particular clonal groups of VRE such as CC-17 are predominantly
associated with infection (9). Therefore invasive isolates are therefore unlikely to
represent the diversity of VRE present in the gastrointestinal tract of patients in the
hospital from which the effluent is sourced and this may relate to the apparent lack of
similarity. MLST or whole genome analyses may reveal relationships that are less
apparent by PFGE as clonal groups such as CC17 may be quite diverse on PFGE (15).

130 From an ecological perspective vanA E. faecium VRE in the aqueous environment
represents a disturbance of natural biodiversity as this is a relatively novel anthropogenic
biological entity related to antimicrobial use. There may be other impacts of microbial
biodiversity that are more difficult to detect. From a human health perspective the
discharge of VRE in to the environment in hospital effluent is a concern as a potential
avenue for spread of VRE into the community however it is difficult to assess its
significance at this time.

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186 composition and persistence of faecal coliforms and enterococcal populations in
Figure 1: Comparison of VRE from environmental and clinical sources

Table 1: Quantitative analysis of effluents and waters for total enterococci and percentage of vancomycin resistant enterococci (VRE)

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Sample size</th>
<th>Total enterococci (MPN$^3$/100ml)</th>
<th>Percentage of VRE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hospital effluent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital A</td>
<td>17</td>
<td>ND$^1$ to 1.1 x 10$^5$</td>
<td>ND$^1$ to 27%</td>
</tr>
<tr>
<td>Hospital B</td>
<td>2</td>
<td>4.7 x 10$^5$ to 1.97 x 10$^7$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td><strong>Municipal effluent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upstream of hospital A</td>
<td>5</td>
<td>3.1 x 10$^5$ to 1.58 x 10$^6$</td>
<td>ND$^1$ to 2.8%</td>
</tr>
<tr>
<td>Downstream of hospital A</td>
<td>5</td>
<td>1 x 10$^6$ to 2.75 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Upstream of hospital B</td>
<td>2</td>
<td>ND$^1$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Downstream of hospital B</td>
<td>2</td>
<td>4.1 x 10$^6$ to 1.45 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td><strong>Wastewater Treatment plant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raw intake</td>
<td>1</td>
<td>1.33 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Post return</td>
<td>1</td>
<td>1.45 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Primary treated</td>
<td>1</td>
<td>1.83 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Aeromon effluent</td>
<td>1</td>
<td>9.8 x 10$^5$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Final treated effluent</td>
<td>13</td>
<td>9.7 x 10$^5$ to 2.5 x 10$^6$</td>
<td>ND$^1$ to 2%</td>
</tr>
<tr>
<td><strong>Rural Group Water Supplies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K Source water</td>
<td>17</td>
<td>ND$^1$ to 7.93 x 10$^5$</td>
<td>ND$^1$ to 6%</td>
</tr>
<tr>
<td>K Piped water</td>
<td>17</td>
<td>ND$^1$ to 5.16 x 10$^5$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>C Source water</td>
<td>17</td>
<td>ND$^1$ to 5.2</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>C Piped water</td>
<td>17</td>
<td>ND$^1$ to 8.05</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>M Source water</td>
<td>17</td>
<td>ND$^1$ to 7.57 x 10$^5$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>M Piped water</td>
<td>17</td>
<td>ND$^1$ to 2.28 x 10$^6$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Rivers</td>
<td>28</td>
<td>ND$^1$ to 2.42 x 10$^6$</td>
<td>ND$^1$ to 1.5%</td>
</tr>
<tr>
<td>Lakes</td>
<td>8</td>
<td>ND$^1$ to 2.61 x 10$^5$</td>
<td>ND$^1$</td>
</tr>
<tr>
<td>Seawater</td>
<td>29</td>
<td>ND$^1$ to 4.2 x 10$^5$</td>
<td>ND$^1$</td>
</tr>
</tbody>
</table>

$^1$ND = Not detected; $^3$MPN = Most Probable Number; $^*$Limit of detection is 1 x 10$^5$

MPN/100ml for untreated effluent, 1 x 10$^7$ MPN/100ml for treated effluent, and 1 x 10$^7$

MPN/100ml for seawater. $^1$ND = not detected
Antimicrobial Agents and Chemotherapy

Production of KPC-2 Carbapenemase by an Escherichia coli Clinical Isolate Belonging to the International ST131 Clone

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Production of KPC-2 Carbapenemase by an Escherichia coli Clinical Isolate Belonging to the International ST131 Clone

The rapid international dissemination of Klebsiella pneumoniae carbapenemase (KPC)-producing organisms is of major concern. Of the 13 variants of KPC described to date, KPC-2 is the most widely reported and disseminated. KPC-producing isolates of K. pneumoniae have reached epidemic proportions in the United States, Israel, and Greece, with increasing reports from other European countries (1, 2, 7, 8).

Escherichia coli O25b:H4-ST131 is a very successful pandemic uropathogenic clone associated predominantly with community-acquired antimicrobial-resistant infection. The close association between the extended-spectrum β-lactamase (ESBL) CTX-M-15 and E. coli ST131 has been implicated in the international dissemination of this enzyme (2, 12). We report the KPC-2 carbapenemase in an isolate belonging to the ST131 clone; this has not been reported previously.

E. coli 490995 and K. pneumoniae 490995.1 were isolated in April 2010 from the urine of an 84-year-old woman. She was an independently mobile long-term resident of an elderly care facility in the Mid-Western region of Ireland, with no history of foreign travel or admission to hospital in the 6 months prior to isolation of these organisms. She had received four courses of amoxicillin-clavulanic acid, two courses of quinolones (ofloxacin and ciprofloxacin), and two courses of cephalosporins (cefuroxime and cefixime) in the previous 6 months for treatment of lower respiratory tract and urinary tract infections. Screening using rectal swabs at the time of isolation of these organisms and 6 months later did not identify carriage of a carbapenemase producer among the seven other residents sharing her room.

The meropenem, ertapenem, and imipenem MICs for E. coli 490995 were >16 μg/ml, and 16 μg/ml, respectively, as determined by agar dilution, and the isolate was susceptible to chloramphenicol, minocycline, amikacin, and kanamycin and resistant to ampicillin, ceftazidime, cefotaxime, cepodoxime, cefoxitin, aztreonam, amoxicillin-clavulanic acid, piperacillin-tazobactam, tetracycline, sulfonamides, streptomycin, gentamicin, ciprofloxacin, nalidixic acid, and trimethoprim by the CLSI disk diffusion method (4). The meropenem, ertapenem, and imipenem MICs for K. pneumoniae 490995.1 were >32 μg/ml, >16 μg/ml, and 64 μg/ml, respectively, and the isolate was susceptible to only 3 of the 19 antimicrobial agents screened: streptomycin, gentamicin, and tetracycline. Both isolates were confirmed as carbapenemase producers by the modified Hodge method of the CLSI, and the KPC enzyme was indicated by a commercial synergy test (Rosco Diagnostica, Taastrup, Denmark). PCR and sequencing confirmed that both isolates harbored blaKPC-2, blaTEM-1, and blaCTX-M-15 (6, 13, 14); K. pneumoniae 490995.1 additionally harbored blaSHV-12. E. coli 490995 was confirmed to belong to the sequence type 131 (ST131) clonal group by PCR (3) and multilocus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli). Both isolates harbored four plasmids ranging in size from 7 to 190 kb. A 120-kb IncFIIK2 plasmid was common to both isolates and carried blaKPC-2 in an isoform “a” Tn4401 element (5).

The occurrence of an isolate belonging to the pandemic E. coli clonal group O25b:H4-ST131 that produces a KPC-type carbapenemase has not been reported previously, although NDM-1 and VIM-1 carbapenemases have recently been reported in this clone (9, 10, 11). Given that carbapenemases are vital therapeutic agents for treatment of severe infection and the success with which CTX-M ESBL-producing E. coli O25b: H4-ST131 has disseminated throughout the world, the spread of carbapenemases into this clonal group is a cause of serious concern.

(This work was presented in part at the 21st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID], Milan, Italy, 7 to 10 May 2011.)

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REFERENCES


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†Published ahead of print on 18 July 2011.
Details of Author’s contribution to published papers

All authors discussed the results and implications and commented on each applicable manuscript (refer to appendix 3, 4, 5 & 6) at all stages of each applicable project.

Appendix 3-

Published Paper: CTX-M enzymes are the predominant extended spectrum β-lactamases produced by Enterobacteriaceae in Ireland

<table>
<thead>
<tr>
<th>Author</th>
<th>Contribution</th>
</tr>
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<tbody>
<tr>
<td>Dearbhaile Morris</td>
<td>Designed and performed experiments, analyzed data and wrote the paper</td>
</tr>
<tr>
<td>Fiona Boyle</td>
<td>Performed experiments - Antimicrobial susceptibility testing, PCR and Pulsed field Gel Electrophoresis</td>
</tr>
<tr>
<td>Victoria Buckely</td>
<td>Collection of bacterial isolates</td>
</tr>
<tr>
<td>Li Xu</td>
<td>Carried out dHPLC analysis</td>
</tr>
<tr>
<td>Belinda Hanahoe</td>
<td>Collection of bacterial isolates and supply of clinical data</td>
</tr>
<tr>
<td>Peter Hawkey</td>
<td>Provided control strains and gave technical guidance</td>
</tr>
<tr>
<td>Martin Cormican</td>
<td>Supervised the project and gave technical guidance</td>
</tr>
</tbody>
</table>
Appendix 4-

Published Paper: Enumeration and Characterization of antimicrobial resistant *Escherichia coli* bacteria in effluent from municipal, hospital and secondary treatment facility sources

<table>
<thead>
<tr>
<th>Author</th>
<th>Contribution</th>
</tr>
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<tbody>
<tr>
<td>Sandra Galvin</td>
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</tr>
<tr>
<td>Fiona Boyle</td>
<td>Field collection of samples and performed experiments</td>
</tr>
<tr>
<td>Paul Hickey</td>
<td>Field collection of samples and EPA contact for the project</td>
</tr>
<tr>
<td>Akke Velinga</td>
<td>Performed all statistical analysis on the project</td>
</tr>
<tr>
<td>Dearbhaile Morris</td>
<td>Supervised the project and gave technical guidance</td>
</tr>
<tr>
<td>Martin Cormican</td>
<td>Supervised the project and gave technical guidance</td>
</tr>
</tbody>
</table>

Appendix 5

Published Paper: *Enterococcus faecium* of *vanA* genotype in rural drinking water, effluent and the aqueous environment

<table>
<thead>
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</tr>
<tr>
<td>Paul Hickey</td>
<td>Field collection of samples and EPA contact for the project</td>
</tr>
<tr>
<td>Martina Mulligan</td>
<td>WWTP contact and aided in collection of WWTP samples</td>
</tr>
<tr>
<td>Martin Cormican</td>
<td>Supervised the project and gave technical guidance</td>
</tr>
</tbody>
</table>
Appendix 6

Published Paper: Production of KPC-2 carbapenemase by an *Escherichia coli* clinical isolate belonging to the international ST131 clone

<table>
<thead>
<tr>
<th>Author</th>
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</tr>
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<tr>
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</tr>
<tr>
<td>Catherine Ludden</td>
<td>Performed experiments - Antimicrobial susceptibility testing, PCR, plasmid analysis and Pulsed field Gel Electrophoresis</td>
</tr>
<tr>
<td>Iris Condon</td>
<td>Detected carbapenemase producers and provided bacterial isolates from MWRH, Limerick to the ARME for further analysis</td>
</tr>
<tr>
<td>James Hale</td>
<td>Carried out MLST analysis and conducted all bioinformatics analyses</td>
</tr>
<tr>
<td>Nuala O Connell</td>
<td>Provided clinical expertise and patient/ epidemiological data on isolates from MWRH, Limerick</td>
</tr>
<tr>
<td>Lorraine Power</td>
<td>Provided clinical expertise and patient/ epidemiological data on isolates from MWRH, Limerick</td>
</tr>
<tr>
<td>Teck Wee Boo</td>
<td>Provided clinical expertise, technical guidance and patient/ epidemiological data on isolates from UCHG Galway</td>
</tr>
<tr>
<td>Hiran Dhanji</td>
<td>Performed experiments</td>
</tr>
<tr>
<td>Christian Lavallae</td>
<td>Performed experiments</td>
</tr>
<tr>
<td>Neil Woodford</td>
<td>Provided control isolates and gave technical guidance on the project</td>
</tr>
<tr>
<td>Martin Cormican</td>
<td>Supervised the project and gave technical guidance</td>
</tr>
</tbody>
</table>
Website developed for the ARME Group
Awards and honors received during this PhD thesis

Dear Fiona

On behalf of the group who judged talks at the Infection 09 meeting on November 11th-13th, ICC, Birmingham. I would like to congratulate you on winning the MSD award for the best research on ESBL’s entitled “Characterisation of plasmids associated with a novel beta-lactamase phenotype (Cefepimase) in Salmonella Typhimurium”.

For your prize you will receive £500

We hope that you will use this award for travel to an international or national meeting of your choice.

You can claim your award by contacting our treasurer as below. I would be grateful if you could do this as soon as possible.

Professor Alastair Leanord
Consultant Microbiologist
Microbiology Department
Southern General Hospital
1245 Govan Road
G51 4TF
Alistair_Leanord@gcc.scot.nhs.uk
Once again I would like to thank you for your excellent contribution at the Infection 09 meeting and contributing to the high standard of the presentations.

Kind regards

David Dockrell
BIS Scientific Affairs Secretary

David H. Dockrell MD.
Wellcome Senior Clinical Fellow,
Professor of Infectious Diseases,
Department of Infection and Immunity
University of Sheffield
The Medical School
Room LU107, L Floor
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Tel 44 (0) 114 271 2160 (direct)
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e-mail: d.h.dockrell@sheffield.ac.uk
Invited to become a Science Ambassador for Discover Science and Engineering (DSE) Ireland
Dissemination of research during this PhD thesis

47th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago September 17th - 20th, 2007

Title: Detection of Vancomycin-Resistant Enterococci in Hospital Effluent in Ireland.  
F. Boyle, S. Galvin, D. Morris, P. Hickey, M. Cormican

Federation of Infectious Societies Conference, Cardiff 28th-30th November 2007

(A) Title: Occurrence of ESBL producing E.coli in rivers in Ireland.  
Fiona Boyle, Dearbhaile Morris, Victoria Buckley, Belinda Hanahoe, Martin Cormican  
S. Galvin, F. Boyle, D. Morris, P. Hickey, M. Cormican

(B) Title: Predominance of CTX-M extended spectrum β-lactamase producing Enterobacteriaceae in Ireland.  
F. Boyle, S. Galvin, D. Morris, P. Hickey, M. Cormican

Society of General Microbiology AGM, Belfast 30th - 31st August 2007

Title: Screening for antimicrobial residues and antimicrobial resistant E.coli and Enterococci in effluent and waters from various sites.  
F. Boyle, S. Galvin, D. Morris, P. Hickey, M. Cormican

European Scientific Conference on Applied Infectious Disease Epidemiology, Stockholm 18th -20th October 2007

Title: Occurrence of antimicrobial residues and antimicrobial resistant organisms in waters and effluent from a number of sites  
S. Galvin, F. Boyle, D. Morris, P. Hickey, M. Cormican
Federation of Infectious Societies Conference, Cardiff 28th-30th November 2007

(A) **Title:** Occurrence of ESBL producing *E.coli* in rivers in Ireland.
*S. Galvin, F. Boyle, D. Morris, B. Hanahoe, V. Buckley, P. Hickey, M. Cormican*

(B) **Title:** Predominance of CTX-M extended spectrum β-lactamase producing *Enterobacteriaceae* in Ireland.
*D. Morris, F. Boyle, B. Hanahoe, V. Buckley, P. Hickey, M. Cormican*

European symposium on water borne pathogens in surface water (SWAP), Luxembourg April 19th-20th 2007.

**Title:** Observation of antimicrobial resistant *E.coli* (including ESBL producers) in outflow from a waste water treatment plant.
*S. Galvin, F. Boyle, D. Morris, P. Hickey, M. Cormican*

Interscience Conference on Antimicrobial Agents and Chemotherapy, Munich 31st March-3rd April, 2007

**Title:** Observation of antimicrobial resistant *E.coli* (including ESBL producers) in outflow from a waste water treatment plant.
*S. Galvin, F. Boyle, D. Morris, P. Hickey, M. Cormican*

18th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona 19th –22nd April 2008

(A) **Title:** Prevalence of the tetA gene and class I and class II integrons in environmental isolates of *E. coli*
*D. Morris, F. Boyle, J. O’Connor, G. Devane, N. DeLappe, G. Corbett-Feeney, M. Cormican*

(B) **Title:** Prevalence of plasmid mediated AmpC beta-lactamases in non-typhi *salmonella* from Ireland
(C) Title: Multi-drug resistance pattern of clinical isolates of \textit{Salmonella enterica}  

\textbf{American Society of Microbiology 108\textsuperscript{th} General Meeting, Boston June 1\textsuperscript{st}- June 5\textsuperscript{th} 2008}  

(A) Title: Prevalence of the tetA gene and class I and class II integrons in environmental isolates of \textit{E. coli}  

(B) Title: Prevalence of plasmid mediated AmpC beta-lactamases in non-typhi \textit{salmonella} from Ireland  

(C) Title: Multi-drug resistance pattern of clinical isolates of \textit{Salmonella enterica}  
Presented by: Dearbhaile Morris  

\textbf{Infectious Disease Society of Ireland 1\textsuperscript{st} Inaugural meeting, Dublin 11\textsuperscript{th}-13\textsuperscript{th} June}  

(A) Title: Occurrence of Ciprofloxacin resistance in \textit{E.coli} isolates of environmental origin.  
S. Galvin, F. Boyle, D. Morris, M. Cormican.  

(B) Title: An evaluation of phenotypic and molecular methods for typing a collection of \textit{Salmonella enterica} serovar Typhimurium. (Hunter’s Discrimination Index).  
F. Boyle, D. Morris, M. Cormican
(C) Title: Activity of Tigecycline against Extended Spectrum β-lactamase (ESBL) producing Enterobacteriaceae
F. Boyle, D. Morris, V. Buckley, B. Hanahoe, M. Cormican

Society of General Microbiology AGM, Harrogate UK 30\textsuperscript{th} March -2\textsuperscript{nd} April 2009

(A) Title: An evaluation of phenotypic and molecular methods for typing a collection of Salmonella enterica serovar Typhimurium. (Hunter’s Discrimination Index).
F. Boyle, D. Morris, M. Cormican

(B) Title: Activity of Tigecycline against Extended Spectrum β-lactamase (ESBL) producing Enterobacteriaceae
F. Boyle, D. Morris, V. Buckley, B. Hanahoe, M. Cormican

19\textsuperscript{th} European Congress of Clinical Microbiology and Infectious Diseases, Helsinki
16\textsuperscript{th}-19\textsuperscript{th} May 2009

Title: Phenotypic and genotypic analysis of a novel extended-spectrum β-lactamase phenotype (cefepimease)
F. Boyle, D. Morris, S. Kariuki, G. Revathi, M. Cormican

3\textsuperscript{rd} Symposium on Antimicrobial Resistance in Animals and the Environment 1 - 3
June 2009 Tours (ARAE 2009)

(A) Title: First Report of Extended Spectrum β-Lactamase producing Salmonella Kentucky isolated from Poultry in Ireland

(B) Title: Occurrence of the fluoroquinolone-modifying acetyltransferase gene aac(6\textsuperscript{'})-Ib-cr in E. coli isolates of environmental origin.
S. Galvin, F. Boyle, C. Ludden, D. Morris, M. Cormican
(C) **Title:** Investigation of the molecular basis for vancomycin resistance in environmental isolates of *Enterococci* in Ireland.

*S. Galvin, F. Boyle, D. Morris, M. Cormican*

**Infectious Disease Society of Ireland 2^{ND} ANNUAL meeting 11^{th}-12^{th} June Dublin 2009**

(A) **Title:** Predominance of CTX-M among Extended-Spectrum β-Lactamase producing *Enterobacteriaceae* in Ireland

*D. Morris, F.Boyle, V.Buckley, C.Morris, L. Xu, B. Hanahoe, F. Higgins, P. Hawkey, M.Cormican*

(B) **Title:** First Report of Extended Spectrum β-Lactamase producing *Salmonella* Kentucky isolated from Poultry in Ireland


(C) **Title:** Phenotypic and Genotypic analysis of a novel ESBL phenotype (cefepimase)

*F. Boyle, D. Morris, S. Kariuki, G. Revathi, G. Corbett-Feeney, M. Cormican*

(D) **Title:** Investigation of the molecular basis for vancomycin resistance in environmental isolates of *Enterococci* in Ireland.

Presented by: Fiona Boyle

*S. Galvin, F. Boyle, D. Morris, M. Cormican*

**3^{rd} ASM Conference on Salmonella: Biology, Pathogenesis & Prevention 5^{th}-9^{th} October Aix de Provence France 2009**

(A) **Title:** Characterisation of a novel ESBL phenotype (cefepimase) in *S. Typhimurium* isolated from Kenya and Ireland

*F. Boyle, D. Morris, S. Kariuki, G. Revathi, G. Corbett-Feeney, M. Cormican*
(B) **Title:** Detection of Extended Spectrum $\beta$-Lactamase producing *Salmonella* Kentucky in Poultry in Ireland


**20th European Congress of Clinical Microbiology and Infectious Diseases, Vienna Austria 10th-13th April, 2010**

**Title:** Phenotypic and Genotypic analysis of a novel ESBL phenotype (cefepimase)

*F. Boyle, D. Morris, S. Kariuki, G. Revathi, M. Cormican*

**Federation of Infectious Societies Conference, ICC, Birmingham 11th-13th November 2010**

**Title:** Characterisation of plasmids associated with a novel beta-lactamase phenotype (Cefepimase) in Salmonella Typhimurium

*F. Boyle, D. Morris, S. Kariuki, G. Revathi, J. Hale, M. Cormican*

**British Soceity for Antimicrobial Chemotherapy-Antimicrobial Resistance Mechanisms Workshop, Birmingham 25th-26th November 2010**

**Title:** OXA-1 is responsible for an Extended Spectrum beta-lactamase phenotype in Salmonella Typhimurium

*F. Boyle, D. Morris, S. Kariuki, G. Revathi, J. Hale, M. Cormican*